Investigation of botrytis bunch rot in wine grapes: the disease cycle and symptom quantification

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BSc MSc (Hons)

Submitted in fulfilment of the requirements for
the degree of Doctor of Philosophy

Tasmanian Institute of Agriculture
University of Tasmania
June 2014

Declarations

Statement of originality

This thesis contains no material which has been accepted for a degree or diploma by the University or any other institution, except by way of background information and duly acknowledged in the thesis, and to the best of my knowledge and belief no material previously published or written by another person except where due acknowledgement is made in the text of the thesis, nor does the thesis contain any material that infringes copyright.

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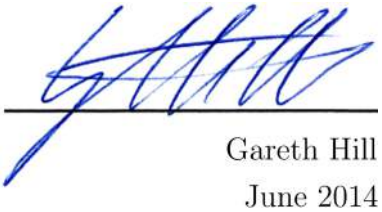

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Statement of co-authorship

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Paper 1 - Use of nitrate non-utilising (*nit*) mutants to determine phenological stages at which *Botrytis cinerea* infection establishes in wine grapes.
Accepted by *Plant Pathology* on March 7^{th}, 2014.

Paper 2 - Visualisation of grape flowers and berries inoculated with green fluorescent protein (GFP)-expressing *Botrytis cinerea*.
Submitted to *Australasian Plant Pathology* on January 30^{th}, 2014.

Paper 3 - Near- and mid-infrared spectroscopy for the quantification of botrytis bunch rot in white wine grapes.
Published in *Journal of Near Infrared Spectroscopy* on January 13^{th}, 2013.

Paper 4 - Comparison of methods for the quantification of botrytis bunch rot in white wine grapes.
Originally formatted for *Plant Disease*.
Accepted by *Australian Journal of Grape and Wine Research* on May 14^{th}, 2014.

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Papers 1 and 2: Katherine Evans and Robert Beresford contributed to the development of the ideas, assistance with methodology and reviewing of the manuscript.

Paper 3: Bob Dambergs contributed to the development of the ideas. Katherine Evans and Bob Dambergs assisted with the methodology. Katherine Evans, Robert Beresford and Bob Dambergs assisted with reviewing of the manuscript.

Paper 4: Katherine Evans, Robert Beresford and Bob Dambergs contributed to the development of the ideas. Katherine Evans and Robert Beresford provided assitance with methodology and reviewing of the manuscript.

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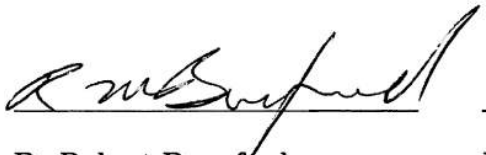

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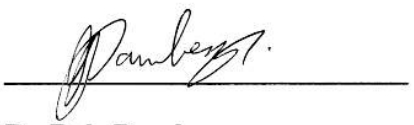

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Abstract

The research described in this thesis investigated various aspects of botrytis bunch rot (BBR), a disease of grapes caused by the fungus *Botrytis cinerea* Pers., including susceptibility of various growth stages of grapevines to infection, *in planta* growth of the fungus and quantification of BBR symptoms. This thesis is presented as a 'thesis by publication' and consists of four papers that have either been published or submitted for publication.

Nitrate non-utilising (*nit*) strains were used to track *B. cinerea* infections throughout the growing season. It was demonstrated that infections occurring at any of three phenological stages of grapevines, flowering, pre-bunch closure (PBC) and veraison, could result in the establishment of latent infections and BBR symptoms at harvest. No single phenological stage led to higher latent incidence or harvest severity than any other stage.

Vitis vinifera berries and flowers were inoculated with green fluorescent protein (GFP)-expressing *B. cinerea* and visualised using fluorescence microscopy, confocal microscopy and brightfield light microscopy. Extensive mycelial growth was observed on flowers, while very little was observed on berries collected and inoculated at PBC. *In planta* growth in mature berries was highly branched and intercellular, growing between the cuticle and the epidermis, with some enlarged hyphae penetrating deeper into the berry tissue. This type of growth is similar to that seen in other host species. Wounding was found to be important for infection, with visibly less hyphal growth observed in non-wounded, mature berries compared with wounded berries.

Infrared spectroscopy was investigated as a potential BBR quantification method and alternative to visual estimation, currently the most commonly used method. Near-infrared (NIR; 800–2690 nm) and mid-infrared (mid-IR; 2510–25770 nm) spectroscopy methods were developed. The spectral range of 1260–1370 nm with Savitzky-Golay smoothing and first derivative pre-processing produced the PLS model with the highest predictive ability in the NIR spectral region. The spectral range of 8760–9520 nm with Savitzky-Golay smoothing and first derivative pre-processing produced the PLS model with the highest predictive ability in the mid-IR spectral region. Both methods demonstrated the potential for spectroscopic quantification of BBR. However, further calibration is required to increase the

accuracy of these models, particularly at low BBR severities, if they are to be considered suitable for use in the vineyard.

The accuracy of visual estimation was evaluated and compared with four other quantification methods: digital image analysis, NIR and mid-IR spectroscopy and quantitative PCR (qPCR). Visual estimation was found to vary significantly between assessors, suggesting that assessor training or correction is required to ensure adequate accuracy and repeatability. Image analysis software (RotBot) was developed to measure BBR severity from digital images of grape bunches using pixel hue. All quantification methods showed significant relationships with visual estimation. Quantitative PCR was the most accurate method but it is too labour intensive to be considered useful for routine use in the vineyard. RotBot was found to be the most suitable alternative to visual estimation, as it is an objective measure and requires no specialised equipment.

This thesis provides new information on the timing of *B. cinerea* infections in vineyards, the *in planta* growth of *B. cinerea* in *V. vinifera* flowers and berries and describes a number of potential alternatives to visual estimation for BBR quantification. It is hoped that this information can be used to improve the control and management of BBR in commercial vineyards as well as enhancing future BBR research.

Acknowledgements

The completion of this PhD involved a long list of people that have helped in one way or another over the past three and a half years. I don't have the space to acknowledge all of them here, but I would like to highlight some of the people whose help was indispensable during my candidature.

Firstly, I would like to thank my primary supervisor, Kathy Evans. Despite being separated by over 2000 kms of Tasman Sea, she has always been ready to help with regular phone conversations, frequent assistance with experiment troubles and comprehensive chapter reviews. While the word count on her comments may have been higher than that of the thesis itself, they kept my writing well on track and up to the highest possible standard. Her amazing estuary-side house, with Hobart-encompassing views, was also kindly offered as accommodation on each of my annual visits to Tasmania. It quickly became my home away from home, and by extension the Evans family became my family away from family. Her husband Evan's extensive knowledge of beer, and the glasses it fills, gave me an appreciation for the beverage that I use frequently to enlighten and bore my family and friends. I also greatly appreciate him and Kathy acting as shuttle drivers and tour guides during my stays, ensuring I experienced more of Hobart than the inside of a laboratory. It was a privilege to witness Gerald's cycling go from a hobby-like passion to a very real shot at the national team, for which I wish him all the best. His motivation and enthusiasm makes me want to get back on a bike, but not at that level, probably not for those distances and definitely not that early on a Sunday morning. The last member of the Evans family I wish to thank is Angus, my other temporary little brother and kindred spirit. He educated me on the nuances of AFL, accompanied me to my first AFL match, reminded me how to lose at video games and allowed me to quote The Simpsons without fear of judgement. Listening to the surprisingly philosophical musings about life, the universe and everything, filtered through the mind of a footy-mad pre-teen, while losing a game of FIFA is an entirely underestimated means of unwinding after a full day crushing berries and staring at spreadsheets. I will remember my stays at the Evans' home fondly and hope the Evans family will come and stay with us if they're ever on our side of the Tasman.

Next I would like to thank Rob Beresford, my supervisor-in-residence. The substantial difference in proximity meant he was the first response team for all the traumas my experiments suffered. He was able to help me salvage the work where it was needed and refine the rest to get it to the level you will read here in this thesis. As my line manager as well as supervisor, he allowed me dedicate as much time as possible to my

PhD, without being overloaded with extra work. His successful isolation of me during the Psa outbreak in NZ meant I was able to avoid the politics and as a result may still be able to work with my colleagues post-thesis.

The final person I'd like to thank in the role of supervisor is Bob Dambergs. I had barely heard of infrared spectroscopy prior to starting my candidature, so as that was the subject of the first published paper from this thesis, he must have done something right. Bob and Lorraine also kindly welcomed me into their home when I wasn't staying with the Evans'. Their house became my home away from my home away from home. They were kind enough to treat me to good food and great wine. They decided to leave The Apple Isle towards the end of my PhD in search of warmer climes, far warmer in fact. They have returned to their vineyard in South Australia and I hope to visit them soon when the sun is out and the wine is poured.

Outside of the supervisory cabinet were a number of people at Plant & Food Research (PFR), Tasmanian Institute of Agriculture (TIA) and within the University of Tasmania (UTAS) that provided specific help on various aspects of my research. Peter Wright and Glen Turner at the Pukekohe Research Station (PFR) both ensured that my field and glasshouse trials ran as smoothly as they can be held responsible for. Peter was even responsible for establishing a new vineyard block when the land was sold out from under the old one. Matt Templeton (PFR) and Gregor Kolbe (PFR) provided the GFP-expressing *B. cinerea* strain that was so vital in the microscopy work. Ian Hallett (PFR) was the microscopy guru that helped me figure out how to prepare my not-so-flat grape samples and what to do with them to get the best results. Justin Direen and Michele Buntain (TIA) provided essential assistance in the form of sample collection prior to and curing my trips to Tasmania. Assistance is an understatement for the first season's work, since I wasn't even in the country at the time. Thomas Rodemann (UTAS) was yet another highly experienced individual who had to deal with this absolute novice when it came to infrared spectroscopy. I should thank the other student's that inhabited Bob's lab during my work in Hobart, as they provided much needed conversation that the frozen berries and homogenizer simply were not able to provide. Also, I apologise to the undergrads in the teaching lab next door, and thank them for their patience, as I not-so quietly pounded the frozen berries with a mallet. I hope their education didn't suffer on account of me.

There are also people at other organisations that were of tremendous help. The late Ross Beever (Landcare Research) was a large part of the development of the infection trials and not only provided the *nit* mutants I used in these trials, but also tips on *B.*

cinerea culturing and spore harvesting that were invaluable for much of my research and will continue to be so in the future. Karyn Hoksbergen (Landcare Research) picked up where Ross sadly left off, and was a friendly face and a great help in getting the strains cultured. John Mackay (DNAture) provided help with qPCR primers and probes, beyond what was required of him. He offered his assistance on his own accord, and I can only assume that much of the smoothness with which the qPCR experiments ran can be attributed to him. I would also like to thank my many Tasmanian vineyard co-operators from Brown Brothers, Craigow Vineyard, Derwent Estate, Josef Chromy and Pooley Wines. They allowed me not only to take what I needed without question, but also to spend a number of sunny days on some of the most picturesque vineyards in Australasia and call it work. Manfred Ingerfeld (University of Canterbury) and Barry O'Brien (University of Waikato) were crucial in getting results from the confocal microscopy work. Although they were only there to oversee my use of the microscope, they helped more than they were obliged to and seemed genuinely disappointed when things didn't go as well as I'd hoped.

Last, but without a doubt the opposite of least, is my loving wife, Bridget. During my candidature she has gone from girlfriend to fiancée to wife to mother of my child. In this time we have renovated a house, sold a house, bought a house, planned a wedding, cruised the Pacific and renovated a house again. Now we are days away from the birth of our daughter. All this has proven a fantastic distraction from the failed experiments, seemingly endless writing and looming deadlines. I like to think I don't bring my work home with me, but she might disagree. I won't say that I couldn't have done this without her, but when I think of how miserable the past three and a half years would have been in her absence, I'm eternally grateful that I didn't have to.

List of abbreviations and symbols

ABTS	2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)
FAM	6-Carboxyfluorescein
ANCOVA	Analysis of covariance
ANOVA	Analysis of variance
BBR	Botrytis bunch rot
BCA	Biological control agent
BHQ	Black hole quencher
bp	Base pair
BRAT	Bunch rot assessment trainer
CF	Capfall
ClO_3	Chlorate
cm	Centimetres
CTAB	Cetyltrimethylammonium bromide
R^2/r^2	Coefficient of determination
r_C	Concordance correlation coefficient
°C	Degree(s) Celsius
d	Days
d.f.	Degrees of freedom
dH_2O	Distilled water
DIC	Differential interference contrast
DNA	Deoxyribonucleic acidÂă
DPM	Diffuse powdery mildew
EDTA	Ethylenediaminetetraacetic acid
eGFP	Enhanced green fluorescent protein
g	Gram(s)

GA	Gibberellic acid
GFLV	Grapevine fanleaf virus
GFP	Green fluorescent protein
GLRaV-3	Grapevine Leafroll associated Virus 3
GMO	Genetically modified organism
h	Hour(s)
HCl	Hydrochloric acid
HSV	Hue, saturation, value
$\mathbf{Hue}_{trough}$	Hue (shifted) with the lowest percentage of pixels between Peakrot and Peakhealthy
ICMP	International Collection of Micro-organisms from Plants
LAMP	Loop-mediated isothermal amplification
LBAM	Light-brown apple moth
LFD	Lateral flow device
LSD	Least significant difference
μg	Microgram(s)
μl	Microlitre(s)
μM	Micromolar
E-L	Modified Eichorn-Lorenz growth stage
M	Molar
m	Metre
MEA	Malt extract agar
mg	Milligram(s)
mid-IR	Mid-infrared spectroscopy
min	Minute(s)
ml	Millilitre(s)
MM	Minimal media
mM	Millimolar
mm	Millimetre
nit	Nitrate non-utilising mutant
n	Number of samples

N_2	Nitrogen
ng	Nanogram(s)
NIPALS	Non-linear iterative partial least squares algorithm
NIR	Near-infrared spectroscopy
nm	Nanometer(s)
NO_3	Nitrate
NSW	New South Wales
NZ	New Zealand
ONFIT	Overnight freezing and incubation technique
P	*P*-value
KNO_3	Potassium nitrate
PBC	Pre-bunch closure
PC	Pathogen coefficient
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
$Peak_{healthy}$	Hue (shifted) for the peak identifying the part of a hue distribution attributable to healthy tissue
$Peak_{rot}$	Hue (shifted) for the peak identifying the part of a hue distribution attributable to diseased tissue
PET	Polyethylene terephthalate
pH	Potential hydrogen
PLS	Partial least squares
ppm	Parts per million
PVC	Polyvinyl chloride
PVP	Polyvinylpyrrolidone
$Pxl_{healthy}$	Pixels corresponding to healthy tissue
Pxl_{rot}	Pixels corresponding to diseased tissue
Cq	Quantification cycle
qPCR	Quantitative PCR
RMSECV	Root mean square error of cross-validation
RMSEP	Root mean square error of prediction

RPD	Ratio of standard error of prediction to standard deviation
®	Registered trademark
RGB	Red, green, blue
rpm	Revolutions per minute
g	Standard gravity
NaCl	Sodium chloride
NaOCl	Sodium hypochlorite
s	Seconds
sGFP	Synthetic green fluorescent protein
SI	Signal intensity
SO_2	Sodium dioxide
°Brix	Total soluble solids
Tris	Trisaminomethane
™	Unregistered trademark
UK	United Kingdom
USA	United States of America
v/v	Volume per volume
VCG	Vegetative compatibility group
H_2O	Water
w/v	Weight per volume

List of tables

The following list contains shortened captions for each table.

List of figures

The following list contains shortened captions for each figure.

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"I have been surprised by the damages which this disease causes in the places that have the misfortune to be affected with it. And who would fail to see that a plant attacked by the disease becomes murderous to others of this species? Has anyone until now observed contagious epidemics in plants?"

— Henri-Louis Duhamel du Monceau, 1728

1

Introduction

Botrytis bunch rot (BBR) in wine grapes is an economically important disease caused by the necrotrophic fungus *Botrytis cinerea* Pers. (Williamson et al. 2007). This pathogen can infect grape flowers and fruit, resulting in rotten berries and grape bunches that are unsuitable for wine making. Disease management can account for 2–6% of production costs in Australian and New Zealand vineyards, and there is potential for lost production and price penalties or rejection of fruit by wineries if disease severity is above a predetermined threshold (Godden 2000, Scholefield and Morison 2009, Evans 2013). It is necessary to understand how the pathogen interacts with the host and how the disease develops in order to design effective disease management strategies. However, there are aspects of the disease that are not fully understood, such as when and how the fungus infects grape flowers and berries, and what the growth characteristics of the fungus are between infection and symptom development.

It is generally accepted that the fungus can infect grape flowers and enter a latent phase until conditions favour disease symptom development, including rotting of the berries and sporulation (McClellan and Hewitt 1973, Nair *et al.*, 1995, Holz *et al.*, 2003, van Kan 2006). It has been shown that grape flowers can become infected (Viret *et al.*, 2004); however, questions remain about whether or not flowering is the predominant

infection stage and the extent to which infections at flowering can persist to harvest. The time between infection, whenever it may occur, and sporulation is known as the latent period. It is unclear what conditions maintain the fungus in a quiescent state or what physiological state the fungus is in prior to symptom expression.

It is important for grape growers to be able to quantify BBR once symptoms develop in order to both understand crop losses resulting from the disease and for accurate, objective measures of disease during price negotiations with wineries or grape buyers (Evans 2013). Visual assessment is the most widely used method for quantifying BBR because it is cost effective and can provide reliable estimates of BBR severity if the assessors are properly trained and sample size is sufficient (Hill *et al.*, 2010). Infrared spectroscopy is used by wineries to measure quality parameters, such as alcohol and anthocyanin content (Cozzolino and Dambergs 2010, Cozzolino *et al.*, 2011), and has shown potential for quantifying BBR (Scott *et al.*, 2010). Quantitative PCR (qPCR) has been successfully used to quantify *B.* cinerea in wine grapes (Cadle-Davidson 2008, Saito *et al.*, 2013), but has not been directly related to BBR severity or tested with infected, mature grape berries. Quantification of disease severity using image analysis has been demonstrated to be effective for some plant diseases (Bock *et al.*, 2010); however it has not been investigated for quantifying BBR severity.

This thesis begins with a review of the literature that identifies gaps in current knowledge about the infection of wine grapes by *B. cinerea*, *in planta* growth of *B. cinerea* during the latent period and research needed to develop and quantify BBR severity using a standardised, objective method for both practical and research applications. Research objectives were developed from the literature review in the context of research required to allow the wine grape industries in Australia and New Zealand to improve their ability to understand and manage this disease.

Figure 1.1 provides an overview of the chapters and experiments conducted in terms of the aspect of *B. cinerea* or BBR being studied. The work is presented as a 'thesis by publication', comprising four manuscripts, each of which has been submitted to a refereed journal or accepted for publication. Manuscripts are formatted according to the style of the target journal and publication details are listed at the beginning of the chapter. The appendices contain experimental results and additional information that was not suitable for publication but which either addressed the thesis objectives or may inform the design of future experiments.

The thesis concludes with a general discussion of the entire study and how it has expanded the understanding of BBR in wine grapes, including new information about the seasonal timing of *B. cinerea* infection and latency, *in planta* growth in grape berries and quantification of BBR symptoms. Issues arising during the course of the research, future experiments that can expand on the major findings and ways in which this research can benefit both researchers and the wine industry are also discussed.

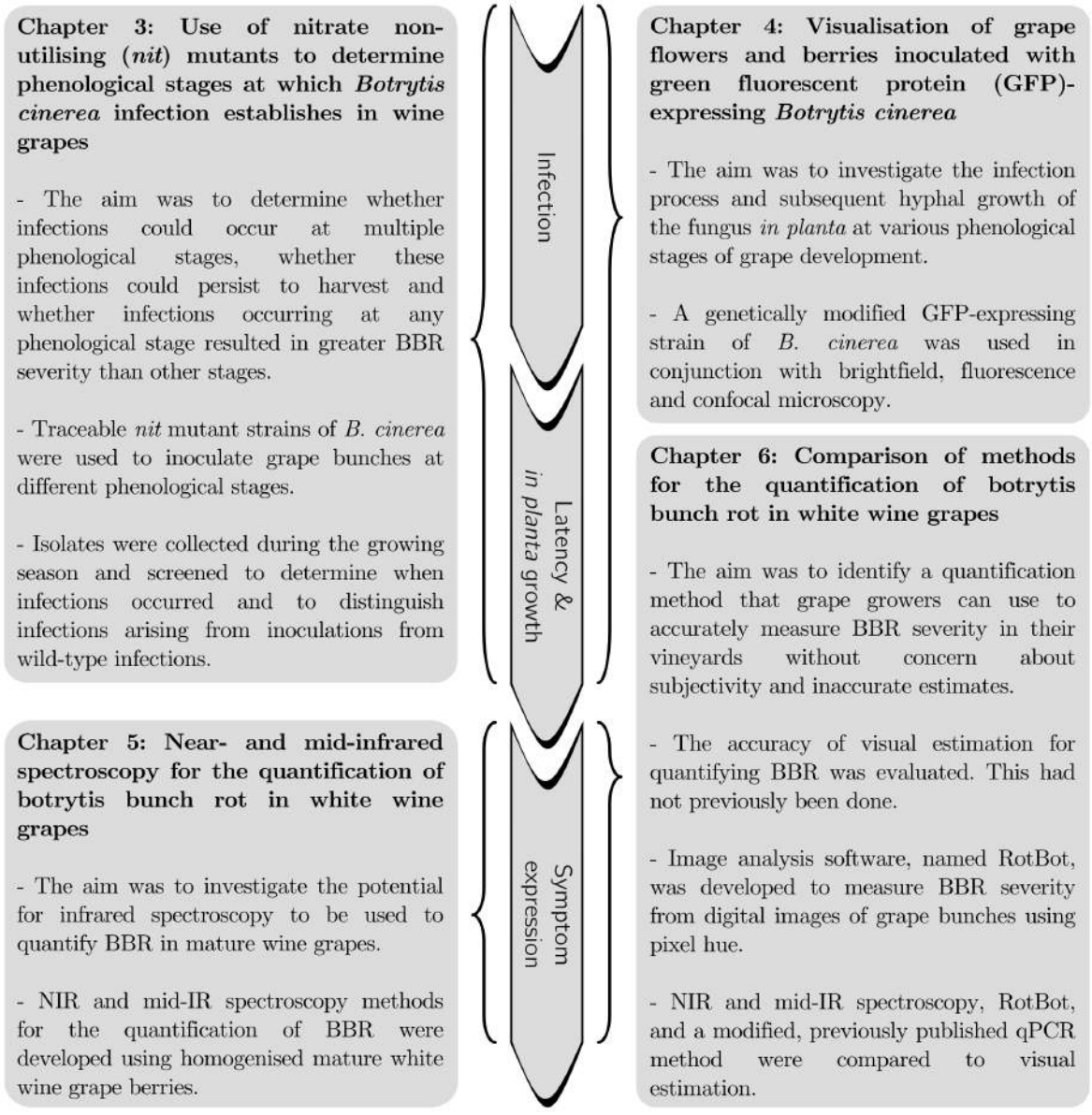

Figure 1.1. Overview of experimental chapters in this thesis and how the work relates to *B. cinerea* and general stages of the botrytis bunch rot (BBR) disease cycle. NIR = near infrared, mid-IR = mid infrared, qPCR = quantitative PCR.

2

Literature review

This review presents a brief history of the wine industry worldwide including some of the diseases affecting the industry, with a particular focus on Australia and New Zealand. A summary of current knowledge surrounding botrytis bunch rot (BBR) follows, including the biology of the causal organism, *Botrytis cinerea* Pers., as well as the impacts, epidemiology and management of the disease. Detection and quantification methods for *B. cinerea* and BBR currently available to grape growers are also outlined.

2.1. Grapevines

2.1.1. History of viticulture and oenology

The grapevine is the most widely cultivated horticultural crop in the world, being grown on every continent except Antarctica (Food and Agriculture Organization of the United Nations, 2012). Grapevine cultivation is believed to have started around 6000–5000 BC, in an area known as Transcaucasia along the eastern shores of the Black Sea (Mullins *et al.*, 2007). It is likely that wine was initially the fortuitous result of the storage of grapes during the winter months, as grapes provide an ideal substrate for fermentation and are

a favourable habitat for yeasts (Mullins *et al.*, 2007). From Transcaucasia, grape growing and winemaking spread into Asia Minor, through the Fertile Crescent and into the Nile delta by 1400 BC, where viticulture is depicted as being well developed in Egyptian mosaics (Mullins *et al.*, 2007). The Phoenicians and Greeks brought viticulture into North Africa, Sicily, southern regions of mainland Italy, Spain and France and by 1000 AD wine drinking had become a well established social custom across most of Europe (Mullins *et al.*, 2007).

2.1.2. Taxonomy of the grapevine

The family *Vitaceae* encompasses all grapevines, including the genus *Vitis*, in which all commercially grown species are found. *Vitis* contains around 60 species that are found mainly in temperate zones of the Northern Hemisphere and are distributed equally between America and Asia (Mullins *et al.*, 2007). *Vitis vinifera* L. is the only species to have originated in Eurasia and is the predominant species used for viticulture in all countries, with the exception of North America where *V. rotundifolia* is the predominant species. There is some disagreement over whether the genus *Vitis* should be divided into two subgenera, *Muscadinia* (*V. rotundifolia* and two other species, commonly referred to as the 'muscadine' grapes) and *Euvitis* (all remaining *Vitis* species), or whether *Muscadinia* should form a separate genus (Conner 2010). As a result, the literature contains both *V. rotundifolia* (Albertini *et al.*, 2002, Andret-Link *et al.*, 2004, Conner 2010) and *Muscadinia rotundifolia* (Diolez *et al.*, 1995, Prado *et al.*, 2009b, Petit *et al.*, 2011) in reference to the same species. Often the term 'muscadine' grapes will also be used to avoid confusion. The more common *V. rotundifolia* will be used here.

2.1.3. Varieties

There are currently around 10,000 varieties of *V. vinifera* grown worldwide. While they are often referred to as 'varieties', the term 'cultivar' is more appropriate as they have been bred from the same species. However, the term 'variety' has become so entrenched in viticulture that it is unlikely to change and will be used here.

More than 100 varieties are grown commercially in Australia (Australian Wine and Brandy Corporation, 2010). The top three varieties accounted for more than 60% of the vineyard area in 2009: Shiraz (27.9%), Chardonnay (18.5%) and Cabernet sauvignon

(17.2%). In New Zealand, more than 50 varieties are grown commercially (New Zealand Winegrowers, 2009); however, the top three varieties accounted for more than 75% of the vineyard area in 2009: Sauvignon blanc (50.7%), Pinot noir (14.9%) and Chardonnay (12.2%).

Varieties can differ in physiological aspects, such as canopy vigour, fruit yield and bunch architecture. These factors can influence the relative susceptibility of the variety to diseases such as BBR (Section 2.3).

In addition to differences among varieties, morphological differences within the same variety can be selected by asexual propagation of genetically identical material to create different clones. These clones retain the predominant characteristics of the variety from which they are selected, although they may possess differences in aspects such as yield potential, fruit composition and sensory qualities (Reynolds *et al.*, 2004).

2.1.4. Viticulture

2.1.4.1. Viticulture in Australia

Grapevines were first introduced into Australia as part of the 'Plants for Settlement' program by the First Fleet in 1788 (Rankine, 2004). The Australian wine industry gained international recognition in 1822 when Gregory Blaxland won the silver medal at the Royal Society of Arts in London for a quarter of a barrel of red wine made at his Parramatta vineyard. James Busby, regarded as the 'father' of the Australian wine industry, emigrated from Britain to Hunter Valley, NSW, in 1824, bringing with him vines from France and Spain, and publishing a book on viticulture (Busby, 1825). Around this time, Matthew Broughton planted the first commercial vineyard in New Town, Tasmania, and in 1827 the first advertisement for his wine appeared in the Colonial Times (Colonial Times, 1827). By the 1850s, German Lutherans had brought viticulture to the Barossa Valley (Mullins *et al.*, 2007). Major increases to the wine industry came after irrigation was established in Mildura in the 1880s and following World War I when the soldier settlement blocks were established on the Riverland in South Australia.

In 1896, the first report of *B. cinerea* being present on grapevines in Australia, specifically the Great Western wine region of Victoria, was published in a Melbourne-based newspaper (Bluno, 1896), although it was initially thought to be beneficial due to the renowned noble wines of Europe. The disease was still confined to Victoria in 1914,

yet its potential negative impact on grape production had been realised (Western Mail, 1914). It is unclear how long it took for the disease to spread to other parts of Australia; however, by the 1980s it had reached Queensland and Western Australia (Barbetti, 1980, Heaton, 1980).

In the 1990s and 2000s, the development of new cool winegrowing regions in south-eastern South Australia, Victoria and elsewhere resulted in a major increase to the wine industry (Figure 2.1). Total planted area of grapevines has decreased in recent years, although the figures remain higher than they were a decade ago. Wine grapes are now produced in all states, with the exception of Northern Territory (Figure 2.2).

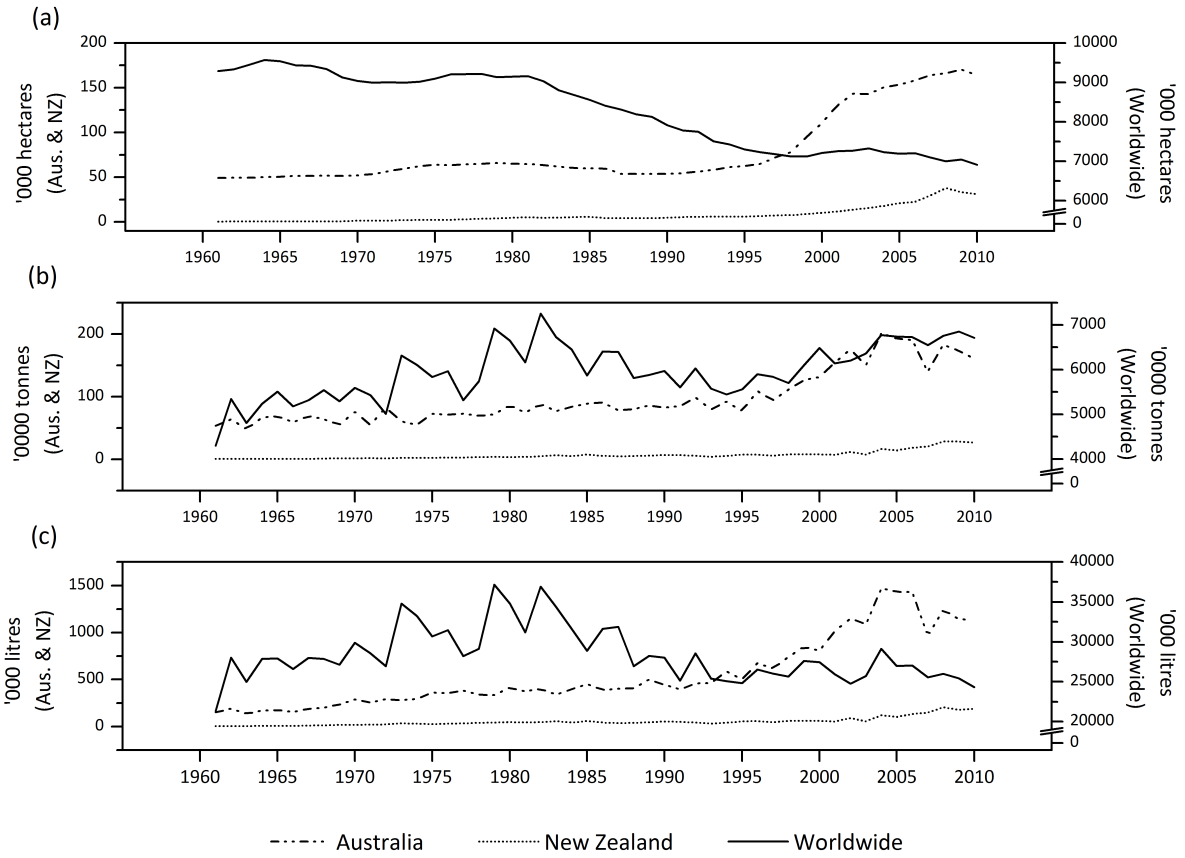

Figure 2.1. Total grapevine planting area (a), grape production (b) and wine production (c) in Australia, New Zealand and worldwide between 1961 and 2010. Data from the Food and Agriculture Organization of the United Nations (FAO; 2012). Grapevine area and grapevine production data do not distinguish between grapevines planted for wine production and those planted for other purposes.

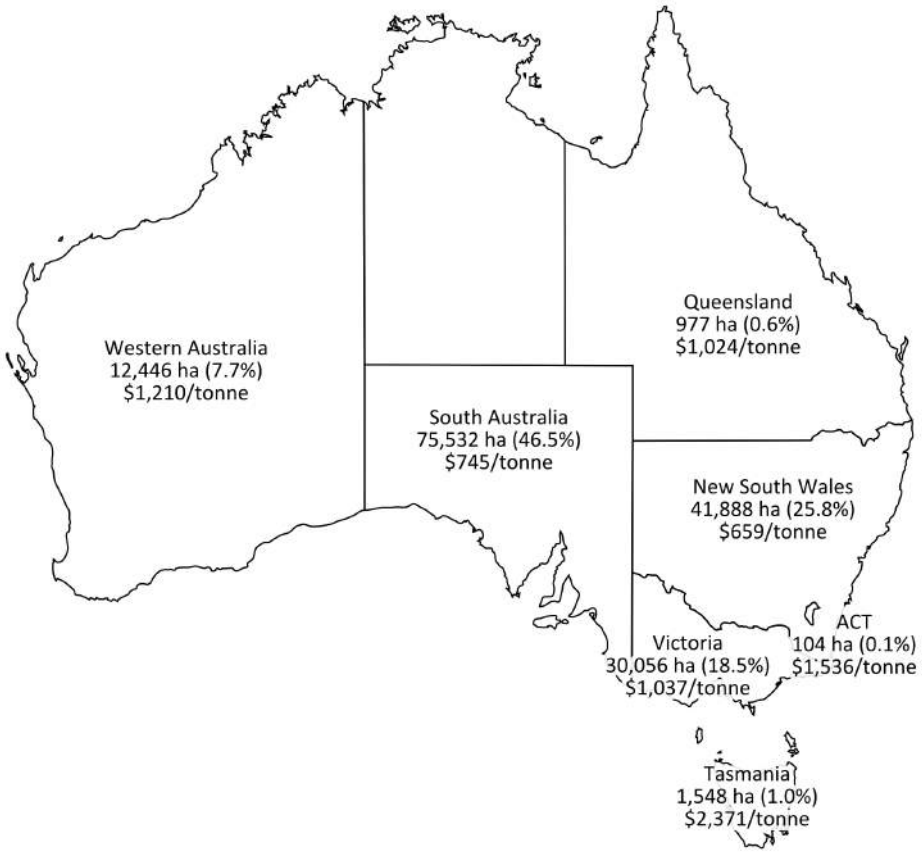

Figure 2.2. Vineyard area and mean grape price per tonne (averaged for all varieties; $AUD) in Australia by state in 2009. Data from the Australian Wine and Brandy Corporation (2010, 2011).

2.1.4.2. Viticulture in New Zealand

The first grape vines in New Zealand were planted in Kerikeri by Samuel Marsden, the Chaplain to the Government of New South Wales (NSW), who brought them over from Port Jackson, NSW, in 1819 (Rankine, 2004). James Busby later brought some of his vines across from Hunter Valley to plant at his residence in Waitangi and produced New Zealand's first known wine in 1840, as mentioned in the diary of Jules Dumont d'Urville, commander of the Astrolabe (Scott, 1964). Hawke's Bay's first vines were planted by the Marist religious order in 1850 and decades later the Corban family opened their winery in Henderson, Auckland, in 1902 (Scott, 1964).

The New Zealand wine industry saw a number of increases and decreases to vineyard area during the 20^{th} century. A lack of labour during World War I and the rising prohibitionist movement resulted in a drop in the area of vineyards in the early part of

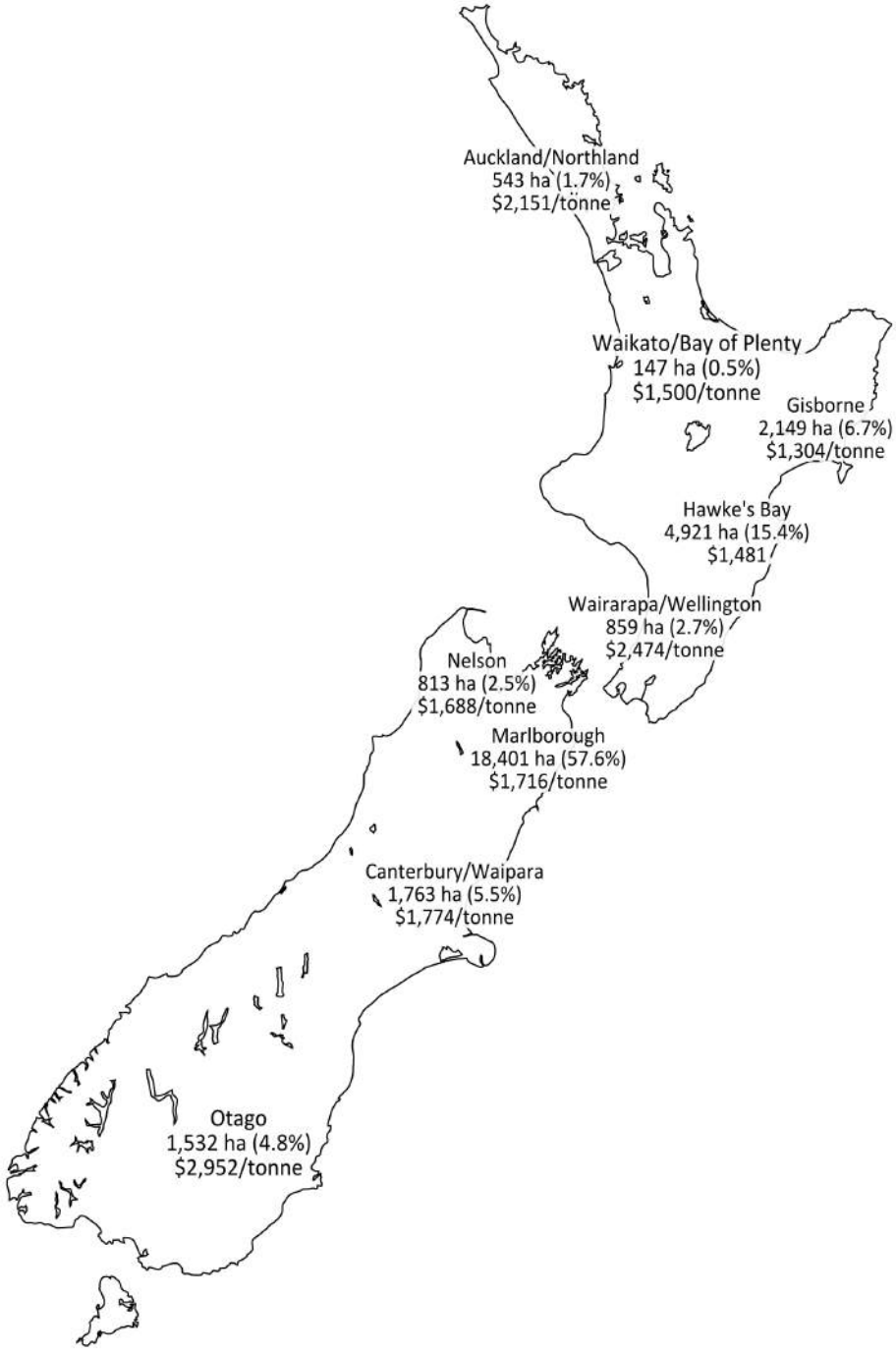

Figure 2.3. Vineyard area and mean price per tonne (averaged across all varieties; $NZD) in New Zealand by region in 2009. Data from New Zealand Winegrowers (2009).

the century (Rankine, 2004). The wine industry began to recover in the 1920s due to a fading interest in prohibition followed by the immigration of a large number of Dalmatian winemakers to Henderson in the early 1930s, and an influx of American soldiers during World War II, which increased demand for alcoholic beverages (Cooper, 1996). There was a steady increase in vineyard area over the 1950s and 1960s, followed by the establishment of new viticulture regions such as Gisborne, Marlborough and Canterbury in the 1970s as part of the world-wide 'planting boom' (Figure 2.1). Increases in the early half of the 1980s were followed by oversupply in the latter half, prompting the government to introduce a national vine removal scheme. Many people used this as an opportunity to remove varieties that were no longer in demand, such as Müller-Thurgau. The scheme resulted in the removal of 25% of the nation's vines (Rankine, 2004). The industry recovered in the 1990s when there was a two-fold increase in vineyard area. This was followed in the 2000s by a three-fold increase, mostly in the Marlborough region, which now accounts for more than half the country's wine production (Figure 2.3).

2.1.5. Rootstocks

Vitis vinifera is renowned for its susceptibility to diseases and pests, particularly to the soil-borne insect pest phylloxera (*Daktulosphaira vitifoliae* Fitch). Phylloxera feeds on the roots and leaves resulting in the formation of galls that envelop the feeding insects (Granett *et al.*, 2001). Galls produced on roots can lead to root necrosis and vine death; however, the reason for this is not entirely clear (Kellow *et al.*, 2004). This insect is commonly referred to as an aphid, however *D. vitifoliae* does not produce any obvious

Table 2.1. Rootstocks commonly used in Australia and New Zealand (May, 1994, Clark, 2004).

Country	Rootstock	*Vitis* species
Australia	Ramsey	*V. champini*
	Shwarzmann	*V. riparia* × *V. rupestris*
	K51-40	*V. champini* × *V. rupestris*
New Zealand	Riparia Gloire de Montpellier	*V. riparia*
	101-14	*V. riparia* × *V. rupestris*
	3309	*V. riparis* × *V. rupestris*

external secretions, unlike aphids, and is placed in the separate family Phylloxeridae (Granett *et al.*, 2001). The pest caused widespread devastation in Europe during the mid-1800s, starting in France, spreading to the rest of the continent and then worldwide (Mullins *et al.*, 2007). It was discovered that phylloxera is unable to form root galls on American *Vitis* spp. This led to the introduction of American vines as rootstocks, which are still the most effective means of dealing with the pest. Rootstocks are thought to provide resistance to phylloxera by reducing the flow of nutrients to the galls, thereby restricting the insect's ability to feed (Granett *et al.*, 2001). This is achieved through developmental characteristics such as rapid growth, early differentiation of the endodermis, early development of the secondary vascular tissues and narrow vascular rays (Pongrać, 1983).

Phylloxera is widespread throughout New Zealand, but in Australia it is only found in small areas of New South Wales and Victoria (King and Buchanan, 1986, Powell *et al.*, 2009). Infected sites have recently been discovered in the Yarra Valley (Powell *et al.*, 2009). The majority of vineyards in Australia, including those in Tasmania, do not use rootstocks and would therefore be highly susceptible to a phylloxera outbreak.

The predominant species used for these rootstocks, *V. rupestris, V. riparia, V. berlandieri* and *V. champini* are referred to as the American vines, due to their North American origins, and have been widely used since the end of the 1800s (Bragato, 1906, Granett *et al.*, 2001). Crossing these species can produce new rootstocks, which are selected not only for phylloxera resistance, but also for good propagation characteristics such as vigour and yield potential and for resistance to nematodes and pathogens, such as BBR (Section 2.3). Rootstocks commonly used in Australia and New Zealand are shown in Table 2.1.

2.1.6. Trellis systems

Another consideration when planting grapevines is the trellis system. This is important as it can affect canopy density, yield, fruit quality, spray penetration and disease impact (Smart and Robinson, 1991). Choice of trellis system also affects the use of mechanisation for trimming, leaf removal, harvesting and winter pruning.

Vertical shoot positioning (VSP; Figure 2.4a) is the most common trellis system in New Zealand, where it is often referred to as the 'standard', as well as being widely used around the world (Smart and Robinson 1991). With VSP, the shoots are trained upwards

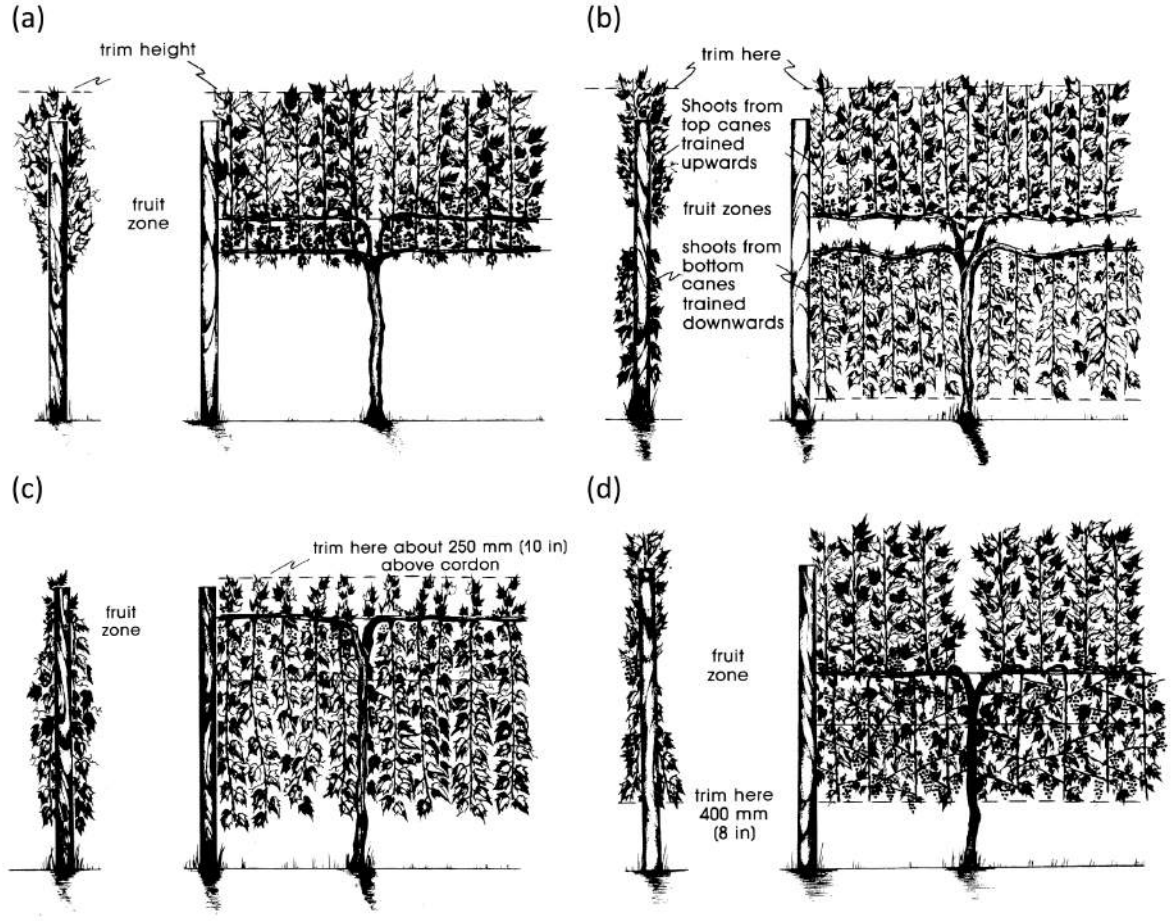

Figure 2.4. Examples of common trellis systems used in vineyards in Australia and New Zealand. (a) Vertical shoot positioned trellis (VSP); (b) Scott Henry trellis; (c) Sylvoz system; (d) Hawke's Bay variant of the Sylvoz system. Adapted from Smart and Robinson (1991).

from the cane on horizontal wires, giving the vineyard a tidy appearance while making it easier to carry out mechanical operations such as leaf removal, spraying, trimming and harvesting. High vigour varieties are prone to shading of grape bunches and interior leaves, which can negatively affect grape development and photosynthetic potential (Perez and Kliewer, 1990, Cartechini and Palliotti, 1995), which can be exacerbated by VSP. In the 1970s, an Oregon grape grower named Scott Henry developed a trellis system (now known as the Scott Henry Trellis; Figure 2.4b) whereby the shoots from the upper canes were trained upwards and the shoots from the lower canes were trained downwards. This method is more time consuming than VSP, and therefore labour costs are increased. However, canopy surface area is increased by approximately 60% and shoot density is halved compared with VSP, increasing fruit exposure. This allows greater spray penetration and increased air flow, both of which are beneficial for disease control

(Smart and Robinson 1991). The Sylvoz system (Figure 2.4c), developed by Carlos Sylvoz in Italy, uses a high cordon with canes either trained downwards or allowed to hang free. While pruning is easier, reducing labour costs, canopy density is increased making the microclimate within vines more conducive to BBR (Section 2.3). A variant of the Sylvoz system was developed in Hawke's Bay in the 1980s (Figure2.4d) to increase canopy surface area thereby decreasing canopy density, lowering disease risk and allowing a greater yield potential.

2.1.7. Phenological stages

Grapevines are perennial deciduous lianes that transition through distinct phenological stages at different times of the year depending on variety, climate and hemisphere. Coombe (1995) described a system for identifying these phenological stages based on modifications to existing systems. They outline 47 phenological stages, labelled E-L (after Eichorn-Lorenz) stages, and group these into five 'macro-stages': shoot and inflorescence development, flowering (Figure 2.5), berry development, ripening and senescence. The terms 'torus' and 'receptacle area' are used interchangeably throughout this thesis, depending on the context. Several E-L stages are of particular importance to grape growers in relation to management actions, such as fungicide applications (Figure 2.6).

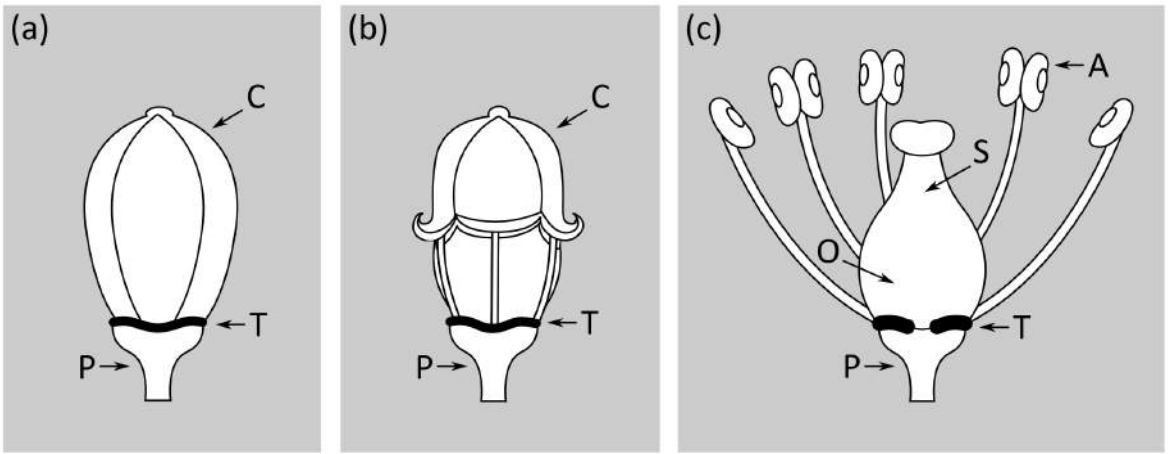

Figure 2.5. Diagram showing the morphology of a grape flower before (a), during (b) and after (c) capfall. A = anther, C = calyptra, O = ovary, P = pedicel, S = style and T = torus or receptacle. Illustration by Ben Galbraith.

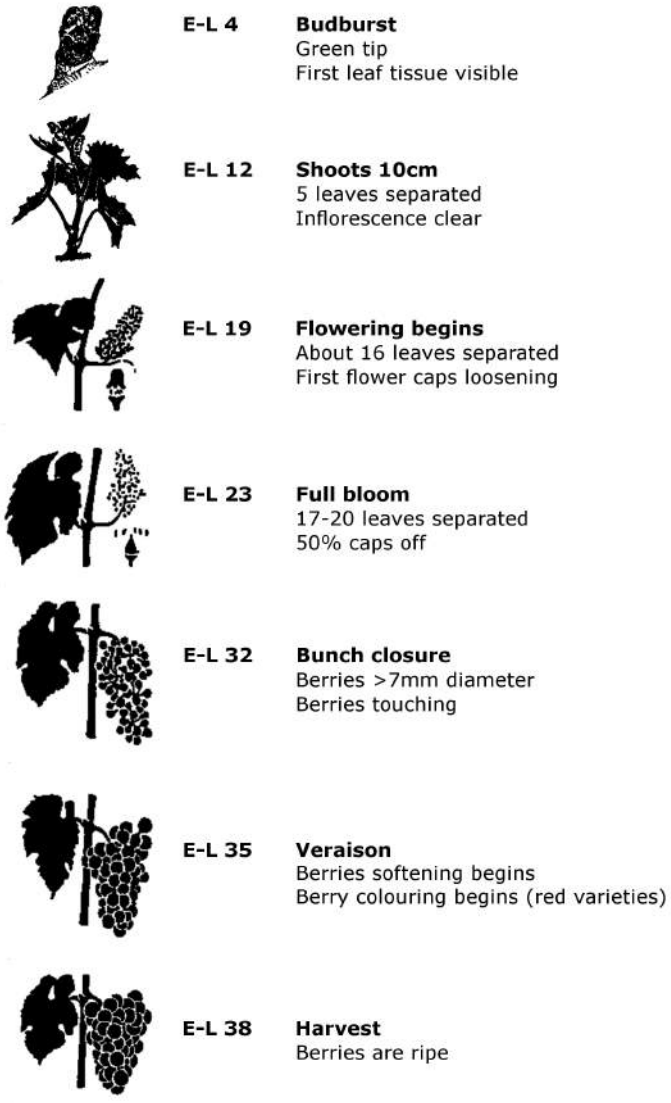

Figure 2.6. Selected E-L (Eichorn-Lorenz) stages that are of particular importance to grape growers for disease control and crop management. Adapted from Coombe (1995).

2.2. Diseases and Pests of Grapevines

Grapevines are susceptible to diseases caused by a wide range of organisms, including viruses, bacteria and fungi (Dhekney *et al.*, 2011).

2.2.1. Viruses

There are a number of virus diseases that can affect grapevines, the oldest and most severe being fanleaf degeneration, often referred to simply as 'fanleaf' (Pearson & Goheen, 1988, Colova *et al.*, 2007). This disease is caused by the grapevine fanleaf virus (GFLV) and is present in nearly all temperate regions in which *V. vinifera* is grown. Fanleaf degeneration was first described in 1841; however, the nematode vector of GFLV was not identified until 1958 and GFLV itself was not identified as the causal organism until the early 1960s (Colova *et al.*, 2007). Infected leaves become asymmetrical and distort resembling a fan, giving the disease its name. In addition to these foliar symptoms, there may be fewer and smaller fruit clusters, berry sugar content can decrease and titratable acidity can increase (Colova *et al.*, 2007).

Another viral disease present in all wine grape growing countries is Leafroll (Pearson & Goheen, 1988). While a number of closteroviruses have been associated with the disease, it is now believed to be predominantly associated with Grapevine Leafroll associated Virus 3 (GLRaV-3; Habili *et al.*, 1995, Charles et al., 2006). The disease is thought to be transmitted by at least three species of mealybug; it can affect vine growth, yield, fruit colour and berry sugar content and can also inhibit photosynthesis by up to 65% (Charles *et al.*, 2006).

With the majority of grapevine virus diseases, the primary management options are control of the vector and the selection and production of virus-free nursery stock (Pearson & Goheen, 1988).

2.2.2. Bacteria

Bacterial diseases are not as prevalent in grapevines as those caused by viruses and fungi. However, one disease that does have a global distribution, and is also widespread amongst plant species, is crown gall. Earliest reports of this disease on grapevines came from France in 1853 (Pearson & Goheen, 1988). While in most plants the causal organism is *Agrobacterium tumefaciens,* in grapevines a separate species, *A. vitis*, has been identified (Ophel & Kerr, 1990). The primary symptom of crown gall disease is a fleshy gall produced on the lower trunk, near the soil line or above grafting wounds, in response to infection. Rootstocks such as *V. riparia* and *V. rupestris* are generally more resistant than *V. vinifera*; however, the most effective method of control is similar to

that of viruses, namely selecting clean planting material and also avoiding mechanical wounding or freeze injuries (Burr & Otten 1999).

Pierce's Disease is a bacterial disease that affects grapevine xylem tissue, resulting in necrotic leaf symptoms and eventually vine death (Pearson & Goheen 1988). The only known treatment for this disease is through control of the vector, the glassy-winged sharpshooter (*Homalodisca vitripennis* (Germar)). The causal organism, *Xylella fastidiosa* Wells *et al.*, is widely distributed throughout the Americas and has only been identified in two other regions: Taiwan and Kosovo (Hopkins & Purcell, 2002). It is considered a biosecurity risk in Europe, Australia and New Zealand, as is the glassy-winged sharpshooter.

2.2.3. Insects

In Australia and New Zealand, the primary insect pest of concern to grape growers is the leafroller moth *Epiphyas postvittana* (Walker), commonly known as light-brown apple moth (LBAM). The pest is native to Australia, but has been introduced into New Zealand. This 8–10 mm moth is mainly active in the early morning and at dusk and is therefore seldom seen in the vineyard (Magarey *et al.*, 1994). Although the moth feeds mostly on the foliage, the major economic damage is caused by feeding on the bunches, which can result in significantly reduced yield (Lo & Murrell, 2000).

In addition to the direct damage caused by LBAM, the larvae of the moth has also been found to transport *B. cinerea* conidia (Bailey *et al.*, 1997), potentially increasing BBR incidence in the vineyard (Section 2.3).

Another leafroller moth, *Ctenopseustis obliquana* (Walker), commonly known as the brown-headed leafroller, is native to New Zealand but not present in Australia. The damage this pest causes to grapevines is similar to LBAM and a brown-headed leafroller infestation is usually treated as though it were an LBAM infestation (Magarey *et al.*, 1994). The relationship between the brown-headed leafroller and *B. cinerea* conidia has not been investigated.

2.2.4. Fungi

Fungi are by far the most prevalent cause of diseases on grapevines (Balasubramaniam, 2011). Many of these diseases are reasonably well understood and established management regimes are in place to minimise the negative impacts they can have on grape production. Fungal diseases on grapevines can be divided into two main groups: the trunk and root diseases, and the fruit and foliar diseases (Pearson & Goheen, 1988).

2.2.4.1. Trunk and root diseases

Fungal diseases of the trunk and roots are less understood than those that affect the fruit and foliage. This may be because they are often not immediately visible until it is too late to manage the disease. Such is the case for Eutypa dieback, a trunk disease caused by the fungus *Eutypa lata* (Pers.:Fr.) Tul. & C. Tul., which is one of the most damaging fungal trunk disease worldwide (Pearson & Goheen, 1988). The foliar symptoms do not appear for at least 4 years after the initial infection occurs. These symptoms include deformation and discolouration of the shoots and reduced growth in young leaves, which often develop necrotic spots and frayed edges. *E. lata* has been identified in both New Zealand and Australia (Sosnowski *et al.*, 2007, Mundy & Manning, 2010).

Another fungal trunk disease that is even more poorly understood, although no less damaging, is Esca. The exact causal organism of this disease complex is unknown (Mundy & Manning, 2010). Symptoms include white, rotted wood fibres in the trunk, sudden wilting of the vine and vascular discolouration of the wood. Multiple species of fungi have been associated with the disease. Although some of these species are present in New Zealand, the symptoms associated with the disease have not yet been seen (Mundy & Manning, 2010). The disease is known to occur in Australia; however, the damage is not as great as that seen in other parts of the world (Edwards & Pascoe, 2004).

A review by Bertsch *et al.* (2013) found that trunk diseases are not as well understood as other fungal diseases, but research in this area is gaining in importance. In 1998, the International Council on Grapevine Trunk Disease was formed in a response to the realisation that research in the area required more focus and collaboration (Govrin & Levine, 2000).

2.2.4.2. Fruit and foliar diseases

Fungal diseases affecting the fruit are of particular importance in the wine industry as they can impact directly on grape production and wine quality. Not only do they remain a concern for grape growers throughout the world, they also hold historical significance in terms of modern viticulture. One such disease is powdery mildew, caused by the fungus *Erysiphe necator* (Schwein.) Burrill. The disease was first described in North America in 1834, however the impact was minor. In the mid-1800s the disease made its way to Europe and began gaining notoriety as it spread throughout the continent, devastating vines and producing 'foul wines' (Pearson & Goheen, 1988). While sulphur was identified as an adequate control method as early as 1847, it was not until 1854, when losses in France had reached nearly 80%, that an application method effective enough to control the disease was developed (Pearson & Goheen, 1988, Large, 2003). After a century and a half, sulphur is still an effective control tactic against powdery mildew in grapevines, particularly when integrated into a spray program with synthetic chemicals (ICGTD, 2011). Fungicide application is focussed in the pre-flowering and flowering period as berries become less susceptible to infection as they mature (Langcake & Pryce, 1976, Ficke *et al.*, 2003). Powdery mildew is now found in most grape growing regions of the world and is one of the most economically damaging diseases for grape growers (Loschiavo *et al.*, 2010). The disease can infect all green tissues of the grapevine. Its presence on leaves is characterised by a whitish-grey, powdery appearance produced by mycelia and conidia on the leaf and fruit surface. Infected petioles or rachi can wither leading to abscission of berries and bunches, while berry infection can result in poor fruit set or splitting of the berries as they develop, facilitating the colonisation of other fungi. Gadoury *et al.* (2007) found that diffuse (non-sporulating) powdery mildew infection (DPM) on the surface of grape berries was associated with significantly increased incidence of BBR. In this same study, percentage insect infestation was also increased in the presence of DPM. Application of insecticides to reduce insect infestation also reduced BBR incidence, suggesting that the relationship between powdery mildew and BBR may be more complex than simply providing more necrotic infection points for *B. cinerea* to infect.

It was only a little over a decade after European viticulturists had learned to manage powdery mildew that they were forced to deal with phylloxera (Section 2.1.5). Unfortunately, it was the solution to this problem, namely the introduction of American vines as rootstocks, that brought with it a new fungal disease, downy mildew (Pearson & Goheen, 1988). The causal organism, *Plasmopara viticola* (Berk. & M.A. Curtis) Berl. & De

Toni, had spread throughout Europe by 1882. In this year, a Professor of Botany at Bordeaux, P.M.A. Millardet, was visiting a vineyard in Médoc when he noticed that vines near the path were free of disease, while the rest of the vineyard had been decimated (Large, 2003). Examining the vines to determine the cause of this phenomenon, he noticed a bluish-white deposit on the leaves. In speaking with the vineyard manager he learned that grape growers in the region routinely coated the vines nearest the path with a poisonous-looking substance to prevent passers-by from eating the grapes. The compound used turned out to be a mixture of copper sulphate and lime. After a number of experiments, Millardet released a formulation of bluestone (copper sulphate) and lime in May of 1885; a formulation that would later become known as '*Bouillie Bordelaise*', or 'Bordeaux mixture' (Large, 2003). Not only was this an effective control against downy mildew, but it remained the most important control of both fungal and bacterial diseases in a variety of crops for over 50 years. Downy mildew is still a problem in winegrowing regions where warm, wet weather occurs during the growing season (Pearson & Goheen, 1988). The disease produces yellow lesions on leaves that are known as 'oil spots' due to their greasy appearance. Severe infection can result in defoliation, which reduces sugar accumulation in the fruit. Infected shoot tips thicken and curl, becoming white with sporulation. As with powdery mildew, berries become less susceptible as they mature, however infections in the rachi can spread into older berries, potentially making downy mildew a problem throughout the season if the primary or first cycle of secondary infection is not prevented (Pearson & Goheen 1988). Downy mildew is found in most wine regions of Australian and New Zealand. The disease can have a major negative impact on grape production, however it does not occur in every season (Brook, 1992, Magarey *et al.*, 1994, Loschiavo *et al.*, 2010).

2.3. Botrytis bunch rot

One of the most important diseases of wine grapes is BBR, caused by the fungus *Botrytis cinerea* Pers.:Fr. (Section 2.3.4), due to both its negative impact on the fruit and potential impact on wine quality. The disease is also known as grey mould. This fungus is a necrotrophic plant pathogen that releases enzymes to decay plant tissue prior to colonisation and absorption of nutrients (van Kan, 2006), unlike biotrophic fungi, which only infect and colonise living host tissue (Mendgen & Hahn, 2002). The host range of *B. cinerea* includes over 200 species and it can affect the roots, shoots, leaves, flowers and fruit (Williamson *et al.*, 2007). Several major crops are affected by this fungus, including

tomato, kiwifruit, roses, strawberry, table grapes and wine grapes (Droby & Lichter, 2004, Williamson *et al.*, 2007).

2.3.1. Disease impacts

Management of BBR can account for approximately 2–6% of vineyard running costs, depending on the disease risk present in a particular region, potentially costing more than NZD$550 per hectare per season (Agnew *et al.*, 2004, Loschiavo *et al.*, 2010, Hoksbergen, 2011). This makes it one of the most economically important disease of grapevines in New Zealand and Australia.

The biggest monetary losses come from crop losses due to BBR as wineries impose price penalties if the amount of BBR in the grapes is above a certain threshold. This threshold can vary depending on the winery and season (Evans 2013). If the grapes are too severely diseased then wineries can reject the grapes altogether. This means that grape growers must invest in effective fungicide regimes and cultural management practices to ensure BBR severity is reduced to below the threshold for the price penalty.

The reason for these penalties being imposed is the effect *B. cinerea* is believed to have on winemaking and the additional costs required to undertake remedial winemaking, if feasible (Godden, 2000). Negative impacts on wine quality are normally attributed to the production of the laccase enzyme by the fungus. This enzyme is relatively stable, and will often persist through the winemaking process, potentially producing undesirable aromas and browning in the must and subsequently the wine (Ribereau-Gayon, 1982, Fregoni *et al.*, 1986). Grapes infected with *B. cinerea* have also been found to have greater titratable acidity and gluconic acid (Fregoni *et al.*, 1986, Perez *et al.*, 1991). While these changes affect the winemaking process by requiring the winemaker to adjust their method to compensate, there are surprisingly few publications relating amount of *B. cinerea* in grapes to undesirable sensory qualities of the resultant wine. *Botrytis cinerea* infection has been partially associated with certain off-aromas (la Guerche *et al.*, 2006). It has also been shown that infection as high as 20% may not be detected by wine tasters (Cassignard, 1977), although the method used by these authors to determine the amount of *B. cinerea*, such as the incidence of berry infection, was not specified. The majority of the literature that focuses on sensory properties of botrytised wine has been more concerned with the positive aspects, such as honey or dried fruit aromas (Sivertsen *et al.*, 2005, Sarrazin *et al.*, 2007).

2.3.2. Botrytis bunch rot symptoms

The first signs of *B. cinerea* infection can sometimes be seen before flowering. Buds and early shoots can turn brown and dry out if infected and leaves can develop reddish brown, irregular necrotic patches, often at the leaf edges (Pearson & Goheen, 1988). Bunch rot symptoms are most often seen after veraison. In white varieties, the berries first turn a pinkish-brown, potentially followed by either berry shrivelling in dry weather or berry splitting in wet weather; the latter appearing to trigger *B. cinerea* to start producing conidia, which appears as a grey mould on the surface of the berry (Figure 2.7). In red varieties the symptoms are more difficult to observe until the fungus sporulates (Figure 2.8), making it more difficult to detect and quantify the disease. Infected berries can also develop a symptom known as 'slip skin', which causes the skin of the berry to slide off when light pressue is applied (Nelson, 1956). If the pedicel or rachis becomes infected, black lesions will appear that can cause parts of the grape cluster distal to the infection to wither (Pearson & Goheen, 1988).

Under certain climatic conditions, *B. cinerea* infection results in what is known as 'noble rot' (Fournier *et al.*, 2013). In this case, *B. cinerea* infection causes the berries to shrivel and increases the sugar content of grapes without the berries completely rotting or falling off. While some of the metabolic differences between noble rot and bunch rot have been characterised (Geny *et al.*, 2003), the specific mechanisms leading to noble rot are not understood. Noble rot is a highly beneficial form of *B. cinerea* infection, producing exceptionally sweet wines such as the Sauternes of France, the Auslese, Beerenauslese and Trockenbeerenauslese wines of Germany, the Tokaj wines of Hungary and the iconic 'Noble One' from the Riverina, NSW, Australia (Bortoli & Mortlock, 2009, Rankine, 2004). These wines are difficult and costly to produce as a lot more attention is paid to the condition of the grapes, in some cases berries are picked individually; therefore, they are sold at a much higher price than other table wines. The higher price also reflects the risks in producing these sweet, botrytised wines, however, as a change in weather can result in the *B. cinerea* infection developing into undesirable bunch rot beyond the point of control, resulting in large crop losses. Efforts have been made in the past to emulate the effects of noble rot by either intentionally inoculating grape bunches or adding *B. cinerea* mycelium directly to the must (De Jong *et al.*, 1968, Ferrarini *et al.*, 2009, Nelson & Amerine, 1956, Nelson & Amerine, 1957, Watanabe & Shimazu, 1976).

Figure 2.7. Typical botrytis bunch rot symptoms on white varieties of wine grapes, seen here on Riesling grapes.

2.3.3. Detection and quantification methods

There is no standardised, objective method for measuring BBR in the vineyard nor relating this disease to the resulting wine quality. The most common technique for quantifying BBR is the visual assessment method. Quantitative polymerase chain reaction PCR (qPCR) and immunological techniques have been developed in recent years; however, not all these techniques are available commercially or readily implemented in practice.

In addition to this lack of a standardised, objective method, the thresholds for incidence of BBR in harvested grapes set by winemakers are relatively arbitrary. While BBR can have negative impacts on wine production, there is no direct evidence that a certain amount of *B. cinerea* in the must will lead to this outcome (Section 2.3.1). The costs of processing BBR affected grapes can be considerable for winemakers; however, varying disease thresholds and associated grape prices mean grape growers can feel unfairly penalised (Godden, 2000). While some of the price penalties may be justified in terms of lost revenue and decreased value of the resultant wine, it is still possible that winemakers set thresholds to provide themselves with a justification for the rejection of grapes in seasons when there is an oversupply. An industry standard BBR threshold, at the very least based on agreement within the industry, combined with a standardised,

Figure 2.8. Typical botrytis bunch rot symptoms on red varieties of wine grapes, seen here on Pinot noir grapes.

objective method of BBR quantification may lead to better agreement between grape growers and wine makers.

2.3.3.1. Visual assessment method

The only method currently commercially-available for BBR severity measurements in vineyards is the visual assessment method. This method requires bunches to be assessed visually and the percentage of infected berries (severity) estimated and recorded. As the only requirements are an assessor and a means of recording data, this method is very accessible to both grape growers and researchers. However, the greatest problem with this method lies in the subjective nature of the assessments, with the potential for substantial variation between assessors (Nutter *et al.*, 2006). A severity assessment key, such as that published by Hill *et al.* (2010), can be used to increase the accuracy of these assessments. Likewise, Bunch Rot Assessment Trainer (BRAT) software is also available to train assessors who may not have much experience or potentially to correct previously collected measurement (Hill *et al.*, 2010). Training and objectivity aside, however, there are further problems with this method. In order to obtain a reliable estimate of BBR severity, around 100 bunches need to be sampled for each vineyard block (Hill *et al.*, 2010). As many vineyards contain multiple varieties and a number of blocks spread

across a large area, this can be very labour intensive, which results in increased operating costs.

Terminology can also cause confusion when discussing this method, particularly between grape growers and researchers, with incidence and severity potentially having different meanings (Hill *et al.*, 2010). Many grape growers in New Zealand use the terms crop loss or percentage crop infection when referring to mean severity for a vineyard or block; when they use the term severity, they are actually referring only to the mean severity of infected bunches, excluding healthy bunches from the equation. This can cause confusion as growers may report a much higher severity for a vineyard as they will not include healthy bunches in the calculation. Standardising a visual detection method would require the elimination of this confusion by clearly defining the terminology.

2.3.3.2. Laccase assay

Much of the concern surrounding BBR is the laccase enzyme produced by *B. cinerea* present in the must (Section 2.3.1). A laccase assay allows enzyme activity to be measured directly and the amount of *B. cinerea* to be inferred from this estimate (Grassin & Dubourdieu, 1989). One of the shortcomings of this method is its inability to work in the presence of sulphur dioxide (SO_2), which is used to sterilise the must. Recently a high-throughput, SO_2-tolerant laccase assay was developed to overcome this problem (Dewey *et al.*, 2008). However, while some correlations have been found between laccase activity and infection by *B. cinerea* (Roudet *et al.*, 1992, Dewey *et al.*, 2008), evidence for the ability of the laccase assay to accurately quantify *B. cinerea* is weak.

2.3.3.3. Lateral-flow devices

Dewey *et al.* (2000) identified a near genus-specific monoclonal antibody to *Botrytis*; they determined that *Botrytis* species other than *B. cinerea* were present in such low numbers in wine juice that any fungus detected by the antibody could be assumed to be *B. cinerea*. They later developed a tube immunoassay using this antibody to quantify *B. cinerea* in grape juice (Dewey & Meyer, 2004). Further work with two companies, one from North America (EnviroLogix Inc.; Portland, ME, USA) and one from the United Kingdom (Forsite Diagnostics Ltd.; Sand Hutton, York, UK), resulted in the development of two lateral-flow devices (LFDs) that could be used on-site for rapid detection of *Botrytis* antigen in grape juice without the need for samples to be sent to an external laboratory, named 'QuickStix™' and 'Pocket Diagnostic', respectively

(Dewey *et al.*, 2008, EnviroLogix, 2008, Forsite Diagnostics, 2011). The use of the LFDs requires one end to be immersed in grape juice. *Botrytis*-specific antibodies bound to gold particles present on the LFD membrane are mobilised as the juice moves along the membrane by capillary action and bind to any *B. cinerea* antigen in the juice. As the juice passes over a line of membrane-bound *B. cinerea*-specific antibodies, particles carrying the *B. cinerea* antigen are trapped, producing a blue line. The presence of more *B. cinerea* antigen will result in a more intense blue line up to a maximum saturation threshold. QuickStix™ can be combined with an electronic reader that measures the signal intensity (SI) of the blue line, providing a numerical quantification of the amount of antigen present in the sample (EnviroLogix, 2008). LFDs provide immediate feedback on the presence of *B. cinerea* in asymptomatic tissue; however, quantification is based on laboratory testing and it has been shown that adjustment of the dilution factor is required to obtain an SI reading when testing juice from grape berries collected from the field (Evans *et al.*, 2010). Without further standardisation, the advantage of the LFDs may be to provide an indication of which samples required further testing with a more accurate quantification method.

2.3.3.4. Molecular detection

In recent years, a lot of focus has been put on developing molecular methods for the detection of plant pathogens using the polymerase chain reaction (PCR; McCartney *et al.*, 2003). Rigotti *et al.* (2002) designed such primers specific to *B. cinerea*, although they later redesigned the primers to allow better specificity (Rigotti *et al.*, 2006). While PCR allows easy and specific detection of *B. cinerea*, this does not solve the problem of quantifying the amount of fungus present. Real-time qPCR allows the amount of deoxyribonucleic acid (DNA) in the reaction to be quantified and through the use of standardisation protocols the amount of fungal mass in the original sample can be estimated. Using the original primers designed by Rigotti *et al.* (2002), qPCR protocols have been developed for quantification of *B. cinerea* (Suarez *et al.*, 2005, Cadle-Davidson, 2008). However, these have not yet been proven to provide accurate measurement in the vineyard. Other protocols have since been developed for detecting latent infections in harvested table grapes (Sanzani *et al.*, 2012) and quantifying BBR severity in wine grapes prior to harvest (Saito *et al.*, 2013). The major disadvantage of qPCR at present is the requirement for expensive laboratory equipment and expertise. This limits the technique to a research tool, as the cost of sending samples for laboratory testing will be too prohibitive for many grape growers. Large wineries may possess the capability to

carry out this type of testing; however, qPCR is unlikely to be widely used in the wine industry due to the difficulty and expense relative to the amount of information gained.

Loop-mediated isothermal amplification (LAMP) is a relatively recent molecular technique that amplifies DNA without the need for thermal cycling (Notomi *et al.*, 2000). The technique has been proven to work for the detection and quantification of *B. cinerea* in rose petals (Tomlinson *et al.*, 2010). The advantage of this technique is that there is no need for expensive PCR equipment and thermal cycling because LAMP can be carried out in a series of water baths. The method is still largely unproven; however, its use as a means of quantifying *B. cinerea* with reasonable accuracy and accessibility outside of the laboratory is promising.

2.3.4. *Botrytis cinerea*

Botrytis cinerea is a fungus belonging to the phylum *Ascomycota*, order *Heliotales* and family *Sclerotiniaceae*. It is the anamorph of *Botryotinia fuckeliana* (de Bary) Whetzel. The genetic connection between the two was controversial until 1953 when isolates of*B. cinerea* were successfully crossed to produce *Botryotinia fuckeliana* apothecia, proving the connection existed (Jarvis, 1977).

The genus *Botrytis* derives its name from the greek word '*botrus*' meaning bunch of grapes, referring to the appearance of conidia attached to conidiophores, and was first proposed by Micheli in 1729. As one of the first genera of fungi ever described, it previously contained a large number of taxa that were either the result of misidentifications or a mistaken concept of the genus. C.H. Persoon assigned *B. cinerea* to the genus in 1801; '*cinerea*' comes from the Latin for grey or ash-coloured, named for the typical grey mould symptoms seen on fruit. It is one of 23 species that remained in the genus when N.F. Buchwald restricted the genus in 1949 (Jarvis, 1977).

The mycelium of *B. cinerea* is made up of septate hyphae that are cylindrical and slightly swollen around the septa (Pearson & Goheen, 1988). Conidia are approximately 15 μm long, smooth, hyaline, elliptical and produced on branched conidiophores with a swollen basal cell (Coley-Smith *et al.*, 1980). Dark, discoid sclerotia can be produced by *B. cinerea* in response to adverse conditions. The fungus can also produce microconidia that are 1–3 μm in diameter and function as spermatia in sexual reproduction, leading to formation of the apothecia of *Botryotinia fuckeliana* (Pearson & Goheen, 1988, Fukumori

et al., 2004). These apothecia are rarely found in nature, but can be readily produced *in vitro* (Beever & Weeds, 2004).

2.3.4.1. Genetic variation in *Botrytis cinerea*

Genetic variation in populations of *B. cinerea* has been used to differentiate isolates and has also been associated with variation in phenotype, such as resistance to certain fungicides.

The first notion that genetic variation could be used to differentiate isolates came when Diolez *et al.* (1995) identified a transposable retroelement in isolates of *B. cinerea*, which they named *Boty*. This transposon contained long-terminal-repeats of 569 bp and was found in 16/17 *B. cinerea* isolates from tomato and 73/79 isolates from various host species. It was not found in *B. cinerea* isolates taken from flax, lentils or peas. A second transposable element was later identified by Levis *et al.* (1997) and was named *Flipper*. The transposon was 1842 bp long and found in *B. cinerea* isolates from tomatoes, grapes and roses. It was realised that *B. cinerea* isolates could be divided into four groups based on the presence or absence of these transposons: *transposa, boty, flipper* and *vacuma* (Table 2.2).

Further work on this genetic variation divided *B. cinerea* into two groups based on resistance to the fungicide fenhexamid, which was present in *B. cinerea* populations before use of the fungicide began (Section 2.3.6.1). This resistance was first found to be associated with distinct polymorphisms in the gene CYP51, which encodes the 14a-demethylase enzyme (Albertini *et al.*, 2002), the target site for a major group of fungicides, the sterol demethylase inhibitors (DMIs). It was later associated with polymorphisms in the gene Bc-*hch*, a homologue of the Nc-*het-c* gene found in *Neurospora crassa* (Fournier *et al.*, 2003). Polymorphisms in Nc-*het-c* were known to determine vegetative incompatibility between strains in *N. crassa*, so it was hypothesised that Bc-*hch* may bestow similar characteristics in *B. cinerea.* This was found to be the case, and Fouriner *et al.* (2003) went on to propose that *B. cinerea* should be divided into two distinct groups: Group I containing strains resistant to fenhexamid, all of which were *vacuma*, and Group II containing those strains susceptible to fenhexamid, which included *vacuma, transposa, boty* and *flipper*.

This research led Leroux *et al.* (2002) to propose that Group I should be a new species with the proposed name *B. pseudocinerea*. Walker *et al.* (2011) confirmed this as a separate species, estimating divergence from a common ancestor occurred 7–18 million

Genetic type	*Boty*	*Flipper*
Transposa	+	+
Boty	+	−
Flipper	−	+
Vacuma	−	−

Table 2.2 The four genetic types of *Botrytis cinerea* based on the presence (+) or absence (−) of the transposons *Boty* and *Flipper*.

years ago rather than a recent speciation event. Minor yet significant differences were found between *B. cinerea* and *B. pseudocinerea* in terms of conidial diameter and growth rate on potato dextrose agar (PDA). However, differences in pathogenicity and host symptoms need to be investigated in order to determine whether the identification of this new species has any consequences for disease management.

A survey of the genetic diversity of *B. cinerea* populations in New Zealand vineyards identified all four genetic types in all regions sampled, with 92% of the total number of isolates collected being *transposa* (Johnston *et al.*, 2013). A similar survey of a single Australian vineyard also found that tha vast majority (76%) of isolates were *transposa* (Cole *et al.*, 2004). Johnston *et al.* (2013) found *B. pseudocinerea* in vineyards in Auckland and Waiapara, but not in Gisborne, Hawke's Bay or Marlborough. There are no published surveys of the distribution of *B. cinerea* genetic types or *B. pseudocinerea* throughout Australia.

2.3.4.2. Inoculum sources

Botrytis cinerea can survive on a wide range of alternative hosts, including grasses and trees species, during the growing season and over winter when grapevine tissues such as leaves and flowers are not present (Cole *et al.*, 2004). Additionally, the fungus can survive on the vine during winter as mycelia on canes or as sclerotia on canes, mummified fruit, tendrils and especially rachides (Nair *et al.*, 1995, Jaspers *et al.*, 2013). Multiple hosts and sites for survival means that *B. cinerea* inoculum, in the form of conidia, is present in the vineyard throughout the growing season (Rodríguez-Rajo *et al.*, 2010).

It is unclear from the literature whether ascospores can also act as a source of inoculum; however, it is unlikely they play a significant role in the infection process as apothecia are seldom found in nature.

2.3.4.3. Mechanisms of infection and disease development

Infection is the process by which a pathogen enters the host tissue. Disease development refers to either the pathogenic process following infection that leads to BBR symptoms or the progress of disease in the host population during an epidemic. The disease cycle encompasses both these processes in addition to the arrival of the pathogen on the host and the eventual production of inoculum and secondary infection. While the epidemiological mechanism for BBR development in grapevines as a whole is still unclear, some aspects of infection and disease development are generally accepted and will be described here.

Infection

Infection of grape flowers and berries by *B. cinerea* can occur from as early as flowering when the first caps are falling, until after veraison when the berries are ripening (Elmer & Michailides, 2004). Although the relative contribution of floral infections to BBR severity at harvest is not fully understood, studies have shown that *B. cinerea* can be found in all floral parts during the early stages of flowering (Nelson & Amerine, 1956, Keller *et al.*, 2003, Viret *et al.*, 2004). These observations at least demonstrate that floral infection can occur, which was not considered to be important for late-season BBR development prior to the 1970s (McClellan & Hewitt, 1973). In strawberries and raspberries, *B. cinerea* colonises either the stylar tissues, followed by hyphal growth down into the ovule or the stamens leading to infection of the receptacle area and subsequently the pedicel and vascular tissue of the berry (McNicol *et al.*, 1985, Bristow *et al.*, 1986). Viret *et al.* (2004) inoculated grape flowers with *B. cinerea* conidia and observed them using light and electron microscopy; they did not find any evidence of *B. cinerea* growing through the style into the ovary. Additionally, inoculation of the stigma did not result in disease expression and Jaspers *et al.* (2013) found no *B. cinerea*-infected stamens in a three-year study of inoculum sources in the vineyard. This suggests that in grapes, if pathogen entry via stamens and stigma is a means of infection, then it is not the only one.

Elmer and Michailides (2004) reviewed mechanisms by which *B. cinerea* infection leads to pre- or post-harvest fruit disease of grapes and other fruit crops. They proposed five âĂŸinfection pathwaysâĂŹ that relate to *B. cinerea* in grapes. However, these can be simplified into three general mechanisms by which mature berries can become infected: 1) infection of floral parts, including the stamens, style, pedicel and âĂŸcap scarâĂŹ (the wound left when the calyptra abscises from the torus), that leads to the establishment of

latent infection (Section 2.3.4.3), 2) infection of maturing berries by conidia or hyphae from infected floral parts, aborted berries or other grape tissues trapped in grape bunches, and 3) infection of maturing berries by conidia from an external inoculum source; either sporulating, infected bunches or other host species within the vineyard. The first two of these involve the infection of grape flowers, and there has been considerable work on how this might occur.

There is evidence that infection can occur near the fruit pedicel. Holz *et al.* (2003) studied sites of infection by incubating naturally-infected grape berries and recording the location on the berry at which *B. cinerea* first grew. They found *B. cinerea* grew near the base and torus of approximately 30% of all berries studied, on the berry cheek in 5% of berries and at the stylar end of only 0.02% of berries. Sanzani *et al.* (2012) found similar results in three varieties of table grapes, where 53–55% of berries yielded infections at the base of the berry, 26–35% on the berry cheek and 11–19% at the stylar end. Although these percentages are higher for the respective berry locations, Holz *et al.* (2003) included leaf and bunch parts, such as rachi, in their calculations. Holz *et al.* (2003) attempted to section the berries before incubation in order to determine whether this was the site of infection or simply an area more conducive to growth out of the berry; however, the resulting skin sections yielded an incidence of less than 1% *B. cinerea* infection.

Infection via the cap scar is another likely infection mechanism. Keller *et al.* (2003) found that when surface-sterilised immature berries were sectioned into three parts, the style, the ovary and the receptacle area, *B. cinerea* was predominantly found in the receptacle area. It was therefore hypothesised that the fungus entered through the cap scar, as this was a significant wound located in the receptacle area. A subsequent study using light and electron microscopy revealed that *B. cinerea* conidia accumulate in the receptacle area (Viret *et al.*, 2004). However, these authors state that the aqueous spore suspension used in the inoculations tended to run off other plant parts and settle on the receptacle area, suggesting that this accumulation of conidia may be a side-effect of the inoculation method rather than a true reflection of the natural infection process. A similar study using naturally infected grape flowers could confirm whether conidia accumulate in the receptacle area under field conditions.

Infection of bunches resulting from infected floral parts and aborted berries trapped within the grape bunch ('bunch trash') is also a potential infection mechanism. Nair *et al.* (1988) found that 28.6% of the bunch trash recovered from within bunches at veraison was infected with *B. cinerea*; 95.5% of this infected material was aborted berries, 3.6%

was calyptrae and the remaining material was made up of leaf, stem and tendril pieces. They did not investigate the relationship between the amount of infected bunch trash and BBR severity at harvest. Jaspers *et al.* (2013) did investigate this relationship across a number of vineyards and found it to be significant in some cases, but generally results were inconsistent. Another study also found that removal of bunch trash significantly reduced BBR incidence, although this was also not the case in both vineyards studied (Wolf *et al.*, 1997). This finding suggests that the relationship between bunch trash and harvest severity is not a constant association. It is most likely influenced by additional factors, such as weather conditions and vine physiology (Section 2.3.5).

While there is evidence supporting all three of these potential infection mechanisms, it is not certain which, if any, plays a more significant role in the epidemiology of the disease. It is possible that all three have equal influence on BBR severity at harvest. Determining the relative importance of each mechanism could potentially enhance the effectiveness of BBR management.

Latency

When *B. cinerea* infection of the flower or young berry occurs, symptoms are seldom seen until the berry ripens. For fungal plant pathogens, the time between infection and symptom appearance is termed the incubation period and the time between infection and sporulation is termed the latent period (Madden *et al.*, 2007). The term latent infection has been used for *B. cinerea* and other fungal fruit pathogens when the fungus, after infection, remains symptomless within the plant tissue for an extended period (Nair *et al.*, 1995); this has also been termed quiescent infection (Cadle-Davidson, 2008). Although the concept of latent infection by *B. cinerea* in grapes was first described in the 1970s (McClellan & Hewitt, 1973), the importance of its role in *B. cinerea* epidemiology is still largely unclear. Nair *et al.* (1995) found a strong, logarithmic relationship (R^2 = 59.9; *P*-value not shown) between the incidence of *B. cinerea* infection in flowers and the incidence of berry infection at harvest, although it is unclear how many vineyards, varieties or years were included in the dataset. The work of Nair *et al.* (1995) has not been repeated. However, Beresford and Hill (2008) found a significant logarithmic relationship between latent infection in berries at pre-bunch closure (PBC) and BBR severity at harvest in multiple vineyards across regions in Australia and New Zealand. While significant, the relationship was not strong enough to allow accurate predictions of harvest severity to be made from latent infection.

It remains difficult to make accurate predictions of harvest severity based on latent infection. One of the biggest questions about this latent period is whether fungal growth is being restrained by plant physiology and biochemical processes, or whether the fungus is simply growing endophytically (within the plant without causing harm) until the conditions are right for necrotrophic growth (van Kan, 2006). Resveratrol, a stilbene, is one of the more abundant phytoalexins produced by *V. vinifera* in response to fungal infection (Langcake & Pryce, 1976, Jeandet *et al.*, 1995). This compound has been found to inhibit *B. cinerea* germination as well as causing several cellular changes including the formation of curved germ tubes and cytoplasmic retraction in the hyphal tip cells (Adrian *et al.*, 1997). Phenolic compounds such as these are more readily found in young berries (Hill *et al.* 1981), suggesting that as berries mature their natural defences are diminished (Hill *et al.* 1981, Pezet *et al.*, 2003). Low soluble solids content (°Brix) in unripe berries may also be a factor in restricting *B. cinerea* growth during the latent period, as it has been shown that the incidence of infection in inoculated berries was greater when berries are at higher °Brix ((Hill *et al.* 1981, Mundy & Beresford, 2007). The activation of latent infections is also unclear, however it has been suggested that increases in humidity, soil water availability and berry nitrogen resulting form pre-harvest rain may play a role (Zitter & Wilcox *et al.*, 2008).

Inoculum production and disease spread

After berry infection, *B. cinerea* may grow out of the berry and produce aerial hyphae that develop conidiophores and conidia. These conidia are a potential source of inoculum.

Conidia are most commonly dispersed by wind and rain splashes (Pearson & Goheen, 1988). Whether or not a secondary cycle of infection occurs does not appear to have been demonstrated in the refereed literature. It is postulated that disease can spread via mycelial growth between berries and onto neighbouring bunches in contact with infected tissue, and by insect transmission (Section 2.2.3).

2.3.5. Epidemiology

Epidemiology is the study of the change in disease intensity in a host population over time and space (Madden *et al.*, 2007). In order to better understand and prevent damage caused by a plant pathogen, it is important to understand the factors that affect the development of the disease epidemic throughout the season. Plant disease

epidemiology was first recognised as a discipline within plant pathology in the 1950s and 1960s (van der Plank, 1963). It requires studying the pathogen, the host and the environment, collectively known as the plant pathosystem, often using the concept of the 'disease triangle', in order to better understand the factors affecting disease development (Campbell & Madden, 1990). Once more is known about these contributing factors, it may be possible to start to make inferences about how the disease will progress in time and space and in some cases to predict disease intensity at the end of the season.

2.3.5.1. Measuring epidemics

An important part of epidemiology is disease assessment and the distinction between incidence and severity. Incidence refers to the number of plant parts that are diseased out of all plant parts sampled (e.g. the proportion of infected grape bunches) and severity refers to the intensity of disease affecting plant parts (e.g. the proportion of infected berries on a bunch). The utility of incidence and severity as measures of disease differs depending on the disease and the purpose for which it is being measured.

Through repeated monitoring of a disease epidemic, using either incidence or severity, it is possible to plot the increase of disease with time and potentially use this information to make predictions about final disease intensity (van der Plank, 1963). These disease progress curves can either be fitted non-linearly (Holb *et al.*, 2005, Li & TeBeest, 2009) or the data can be transformed to obtain a linear fit for ease of analysis (Mohapatra *et al.*, 2008, Craven & Morey, 2011).

Beresford *et al.* (2006) found that BBR severity epidemics follow logistic (sigmoid) growth throughout the season. A linear regression can be fitted by transforming mean severity data using the following logit equation:

$$f(x) = ln\left(\frac{x + 0.1}{100.1 - x}\right) \tag{2.1}$$

where x = BBR severity (Figure 2.9). The logit equation is bound by lower and upper asymptotes, in this case 0 and 100, respectively. Adjusting the upper asymptote can often provide a better fit, however this is only useful when fitting epidemics retrospectively as all data points, including final severity, must be known in order to determine the effect of an asymptote on the goodness of fit (Figure 2.10).

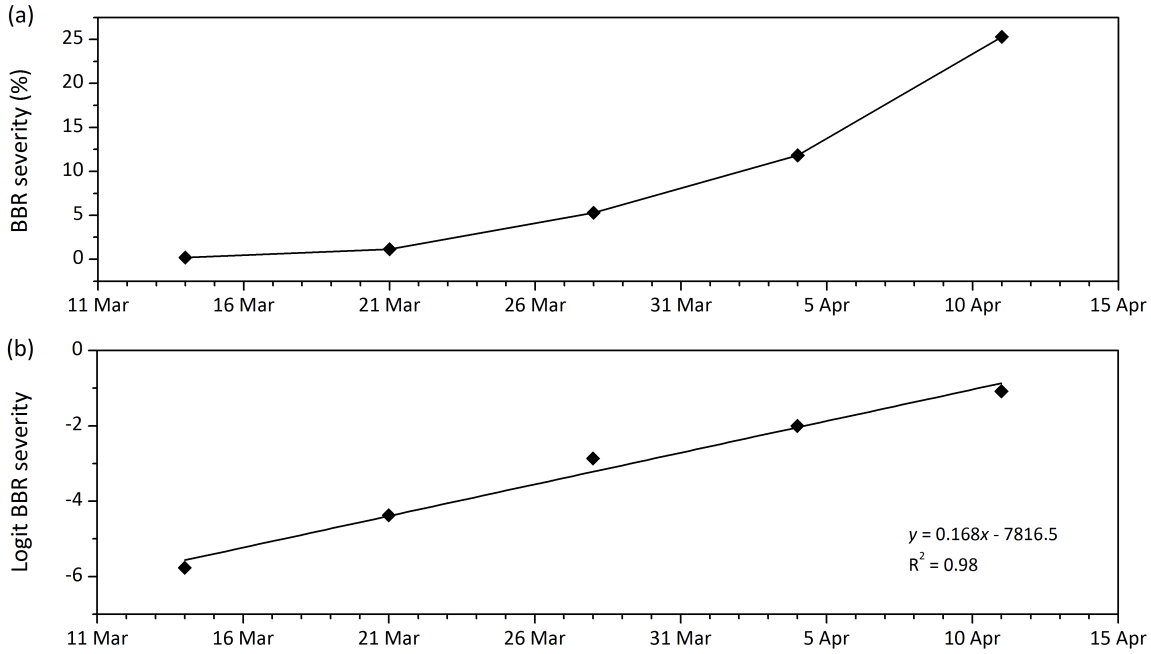

Figure 2.9. Botrytis bunch rot (BBR) severity data from a 2007 field trial in Pukekohe, Auckland (Beresford *et al.*, 2007) showing mean severity for non-treated control plots (a) and logit-transformed mean severities with fitted regression line (b). Regressions use Microsoft Excel dates on x-axis (1 January 1900 = 0).

2.3.5.2. Influence of the host on BBR epidemics

Genes in *V. vinifera* that confer resistance to diseases have been documented for anthracnose, caused by *Elsinoë ampelina*, and for downy mildew (Monteiro *et al.*, 2013, Seehalak *et al.*, 2011), but no such resistance genes have been described for *B. cinerea*. However, changes in susceptibility of grape tissues to *B. cinerea* at different stages of vine development play an important role in the epidemiology of BBR in vineyards. Factors such as phenological stage, canopy density, vigour, grape bunch architecture and variety can all impact the development of the epidemic (Hill *et al.*, 1981, Nair *et al.*, 1988, Vail & Marois, 1991, Pezet *et al.*, 2003, Hed *et al.*, 2009). The relative susceptibility of different phenological stages is not something than can be altered, as this is simply part of the vine's development; as grape berries mature they appear to become more susceptible to *B. cinerea* infection (Section 2.3.4.3). By understanding which stages are more susceptible, however, grape growers can target their management actions to the periods throughout the season that are at a higher risk of infection. Canopy density and vigour can be altered using cultural techniques (Section 2.3.6.3). Although bunch compactness is determined by variety, it is possible to alter compactness through the

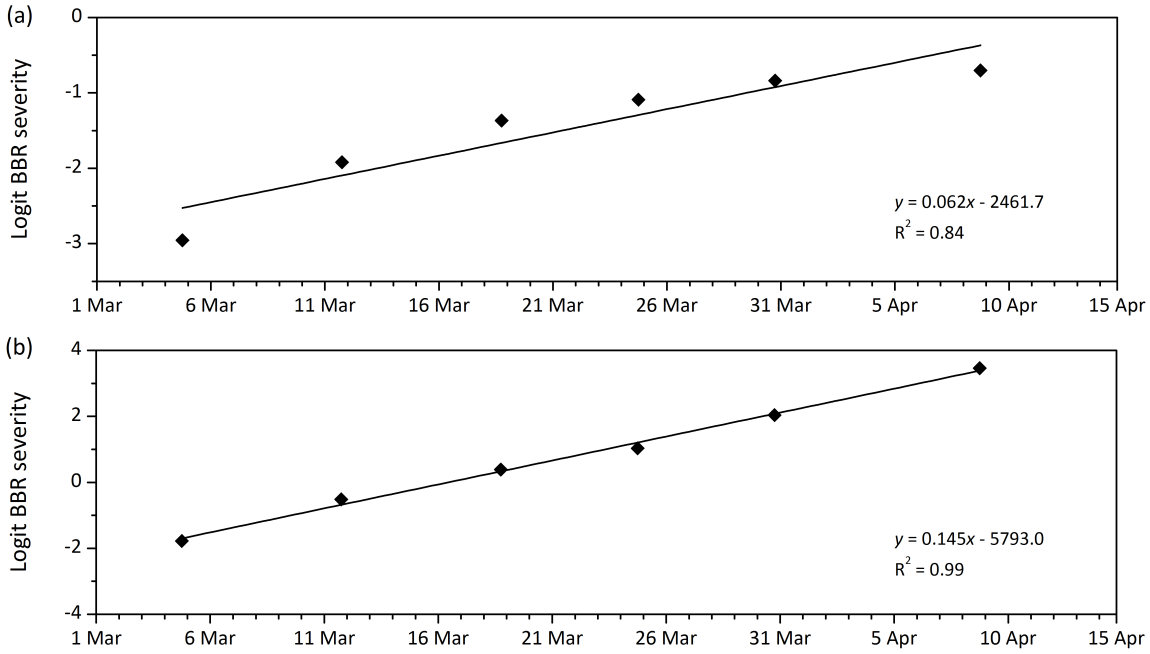

Figure 2.10. Linear regressions using logit-transformed botrytis bunch rot (BBR) severity data from a 2009 field trial in Pukekohe, Auckland (Beresford *et al.*, 2009) using an asymptote value of 100 (a) and an asymptote value of 34 (b). Regressions use Microsoft Excel dates on x-axis (1 January 1900 = 0).

application of gibberellic acid (GA) in order to reduce BBR incidence (Hed *et al.*, 2011). The application of GA has been found to reduce yield (cluster weight; Blaha, 1963, Dass & Randhawa, 1968). While this reduction in yield is not consistent throughout all trials (Ferree *et al.*, 2003, Hed *et al.*, 2011), it is currently enough of a factor to prevent GA being used as a means of reducing BBR risk. Varietal susceptibility results from a combination of the factors mentioned above. Some varieties may have higher canopy vigour, tighter bunch architecture or develop in such a way that more susceptible phenological stages occur during parts of the season where weather is conducive to *B. cinerea* infection. Much of this is a predisposition that cannot be altered; however, choice of variety may require some thought at the time of planting if doing so in a region with a high risk of BBR.

2.3.5.3. Influence of weather and climate on BBR epidemics

Another important factor to consider in the epidemiology of BBR is weather. One of the earliest studies on the epidemiology of BBR in grapes focussed on the optimal conditions for BBR infection and found that incidence of infection of detached grape bunches varied

depending on temperature and duration of wetness (Nelson, 1951). This study found that 12–24 h surface wetness was required for infection with an optimum temperature of 16°C; however, sample size was unclear and statistical analyses were not presented. Other models have since been developed with more rigorous conditions that use a combination of wet periods and temperature as an indicator of *B. cinerea* infection risk (Nair and Allen 1993, Broome *et al.*, 1995, Kim *et al.*, 2007). While it is widely believed that rain events during ripening can cause a significant increase in BBR severity, it is possible that surface-wetness has more of an effect on the rate of disease development. Should a large amount of rain fall within a short time period followed by rapid drying conditions (e.g. sunshine and high wind speed), the actual wetness duration may not be long enough for infection to occur. Conversely, if there is a small amount of rainfall with high relative humidity, surface wetness may persist for many hours. *Botrytis cinerea* has been found to prefer a moist environment of approximately 93% relative humidity, to one where free water can be found on the surface of the berry (Coertze *et al.*, 2001). Therefore, too much rainfall may in fact reduce the likelihood of infection by creating a film of water on the berry surface. It is possible that the belief that heavy rain events drive BBR epidemics comes from grape growers monitoring their vineyard for disease only after significant rainfall events; BBR symptoms would appear to have appeared suddenly, despite symptoms possibly being present at low levels weeks earlier and simply not having been observed.

Beresford *et al.* (2012) have developed an empirical model that combines disease monitoring, linear regressions of logit-transformed disease severity data, vine physiology and weather data in order to provide both potential seasonal BBR risk and an estimate of BBR severity at harvest. This model has been developed into an online decision support tool (http://www.bunchrot.co.nz) that allows grape growers to evaluate the current BBR risk in order to make more informed management decisions.

2.3.6. Disease management

There are a number of methods for the control of BBR on wine grapes. Traditionally this has involved the use of funcigides, but a growing trend towards fungicide residue-free wine production (New Zealand Winegrowers, 2012) is shifting the focus towards biological control agents (BCAs), 'alternative' products (e.g. mineral oils and essential oils) and cultural techniques. Integration of the multiple control measures outlined below is a strategy to manage BBR while minimising fungicide residues. A better understanding

Table 2.3. Fungicide products registered for use against *Botrytis cinerea* in wine grapes in New Zealand for the 2012/13 season (New Zealand Winegrowers, 2012).

Trade Name	Active Ingredient
Amistar®	Azoxystrobin
Bravo®	Chlorothalonil
Captan	Captan
Cobra™	Dimethomorph & chlorothalonil
Copper	Copper ammonium acetate, copper hydroxide, copper oxychloride, cuprous oxide
Folio Gold®	Metalaxyl-m & chlorothalonil
Gem®	Fluazinam
Max Cl™	Metalaxyl & chlorothalonil
Pristine®	Boscalid & pyraclostrobin
Protek™	Carbendazim
Rovral®	Iprodione
Scala®	Pyrimethanil
Sumisclex®	Procymidone
Switch®	Fludioxonil & cyprodinil
Teldor®	Fenhexamid
Teracep®	Peracetic acid & hydrogen peroxide
Thiram®	Thiram
Topsin®	Thiophanate-methyl

of the biology of BBR epidemics and the environmental and host-related factors that influence the epidemic will be vital for the development and integration of new control strategies if growers hope to achieve nil-residue wine production.

2.3.6.1. Fungicides

Types of fungicides

A range of modern synthetic fungicides are used for managing BBR (Table 2.3). Fungicides in the anilinopyrimidine (eg. cyprodinil and pyrimethanil) and phenylpyrrol (e.g. fludioxonil) groups, which were released in the mid-1990s, are currently in widespread use. The fungicide fenhexamid, in the hydroxyanilide group, was released more recently

(Rosslenbroich & Stuebler, 2000). Certain fungicide active ingredients have been deregistered for use in recent years due to either potential health risks or the manufacturer of the chemical not renewing the registration.

A fungicide's mode of action can either be multi-site (broad spectrum), affecting many biochemical processes within the pathogen, or site-specific, affecting a single, well-defined biochemical process within the pathogen; older fungicides tend to be multi-site, while modern fungicides tend to be site-specific (Brent & Hollomon, 2007).

All fungicides used in the wine industry in Australia and New Zealand have a specified pre-harvest interval (PHI). This interval is the minimum length of time allowed between spray applications and the harvesting of the fruit, and can be defined as a number of days or a particular phenological stage (Figure 2.3). PHIs are based on maximum residue limits (MRLs) in the wine, which in turn are set by the country in which the wine is to be sold. Some countries have not set MRLs for certain products, which can prevent exporting to these countries. As the trend towards nil fungicide residues continues, grape growers are left with fewer options in terms of fungicides for managing BBR.

Fungicide resistance

Fungicides can be a very effective means of controlling *B. cinerea* in vineyards. However, frequent use of fungicides with site-specific modes of action has the potential to select for resistant strains within the fungal population (Brent & Hollomon, 2007). As a result of this, it is possible for *B. cinerea* to develop resistance to fungicides if fungicide resistance strategies are not deployed (Martin *et al.*, 2005). These strategies are: 1) limiting the number of times an at-risk fungicide is used each season, 2) mixing at-risk fungicides with broad spectrum fungicides, and 3) alternating each application of an at-risk fungicide with a different type of fungicide (Martin *et al.*, 2005).

One of the earliest examples of resistance being observed in the field was to the benzimidazoles. The first fungicide from this group, benomyl, was released in the 1960s, followed by others such as carbendazim. Strains of *B. cinerea* exhibiting benzimidazole resistance were soon discovered in vineyards around the world and these fungicides were of little use by the end of the 1970s (Beever et al., 1989), leading to their replacement by the dicarboximides (e.g. iprodione and vinclozolin). Because of the rapid development of resistance to benzimidazoles, surveys for resistance to dicarboximides were carried out in various countries (Pommer & Lorenz, 1982). The first signs of resistance were found in New Zealand in 1983 (Beever & Brien, 1983) and later were reported in Australia in

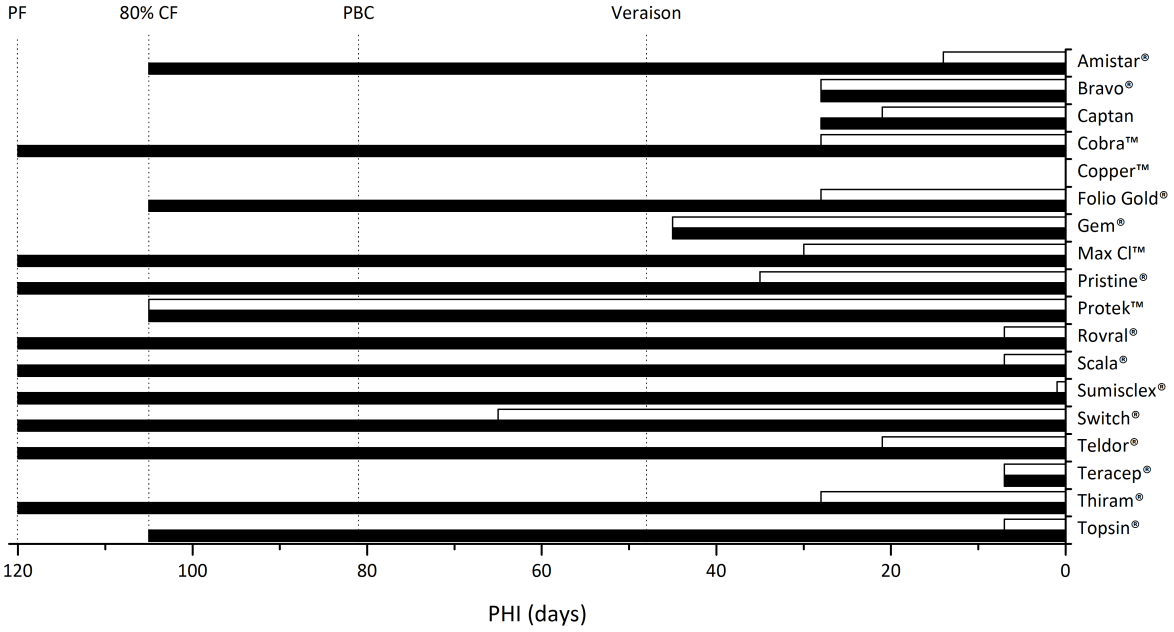

Figure 2.11. Pre-harvest intervals (PHIs) for registered fungicides in New Zealand during the 2012/13 season for both maximum residue limits (MRLs; white bars) and nil residue limits (black bars). PHIs specified as a phenological stage have been converted to number of days based on the mean number of days between phenological stages at 29 vineyards in New Zealand and Australia between 2006 and 2009. Product trade/common names are shown; for active ingredient see Table 2.3. MRLs shown are for the majority of countries in the schedule; Pristine®, Protek™, Rovral®, Scala®, Sumisclex®, Switch®, Teldor®, Teracep® and Thiram® have either greater PHIs or nil residue restrictions in some countries. PF = pre-flowering; CF = capfall; PBC = pre-bunch closure. Data derived from New Zealand Winegrowers (2012).

1985 (O'Brien & Glass, 1986). By the late 1980s, dicarboximide-resistant strains of *B. cinerea* were found throughout New Zealand at such high frequencies that they decreased the effectiveness of this group of fungicides (Beever *et al.*, 1989).

Resistance to more recently introduced fungicide groups has also been reported, including the anilinopyrimidines (Sergeeva *et al.*, 2002) and fenhexamid (Fillinger *et al.*, 2008). In some cases, fungal isolates with resistance, or reduced sensitivity, to a particular fungicide can occur in populations not previously exposed to the fungicide (Suty *et al.*, 1999). While this resistance can be useful for distinguishing strains or for separating genetic groups (Section 2.3.4.1), the potential loss of efficacy is of great concern to both grape growers and agrichemical companies.

2.3.6.2. Biological control agents and alternative products

The move towards residue-free wine production has prompted the recent development of biological control agents (BCAs) and other alternatives to fungicides. The most common BCAs being developed for BBR control are filamentous fungi from the genera *Trichoderma, Ulocladium* and *Gliocladium*, bacteria from the genera *Bacillus* and *Pseudomonas* and yeasts from the genera *Pichia* and *Candida* (Jacometti *et al.*, 2010).

Three BCAs are currently registered for use in New Zealand against BBR in vineyards (Jacometti *et al.*, 2010). *Trichoderma atroviride*, sold under the trade name Sentinel® (Key Industries, 2011), is a saprophytic fungus that restricts the colonisation of *B. cinerea* through competitive exclusion and can also inhibit spore germination (Reithner *et al.*, 2005). *Trichoderma atroviride* was found to reduce *B. cinerea* sporulation on strawberry leaves; however, there was no reduction in the incidence of diseased fruit (Card *et al.*, 2009). *Ulocladium oudemansii* is another saprophytic fungus with a similar mode of action, sold under the trade name Botry-ZEN® (Elmer *et al.*, 2005). The efficacy of *U. oudemansii* against *B. cinerea* is not entirely clear as studies claiming the BCA reduced BBR severity have combined the BCA with fungicides or other products; where the BCA is applied alone there were no statistical mean comparisons performed (Elmer *et al.*, 2005, Reglinski *et al.*, 2005, Reglinski *et al.*, 2010). It is therefore not possible to state definitively that *U. oudemansii* reduces BBR severity. Finally, *Bacillus subtilis* is a bacterium sold under the trade name Serenade® MAX (AgraQuest, 2011). While the efficiency of control of BBR by Serenade® MAX in wine grapes is unclear, control in table grapes has been demonstrated (Elmer & Reglinski, 2006). Furuya *et al.* (2011) suggested that *B. subtilis* inhibits *B. cinerea* infection of wine grapes; however, their sample size was small and only the percentage of rotted bunches was measured. *Botrytis cinerea* infection was not separated from infection by *Colletotrichum gloeosporioides* (Penz.) Sacc.

Alternative products are also available for the control of BBR, including essential oils, mineral oils and plant defence stimulants (Jacometti *et al.*, 2010). Many essential oils have been shown to inhibit *B. cinerea* growth *in vitro*, however only thyme oil has been demonstrated to have effective control in the vineyard (Walter *et al.*, 2001). Mineral oils, such as liquid paraffin, have also exhibited control both as a sole control agent and combined with fungicide use (Dell *et al.*, 1998); however, these materials also show negative side effects such as a reduction in photosynthesis and a decrease in soluble solids (Jacometti *et al.*, 2010).

Plant defence stimulants are intended to boost the grapevine's natural defences (Gozzo & Faoro, 2013). There are a number of commercially-available products on the market. Chitosan, a linear polysaccharide often obtained from the shells of crabs, is marketed under the trade name ARMOUR-Zen®. This product has been found to have a dual mode of action against *B. cinerea* in wine grapes, both inducing the plants natural defences (increased phytoalexin production, chitinase activity and β-1,3-glucanase activity) and offering direct antifungal activity (Aziz *et al.*, 2006, Reglinski *et al.*, 2010). The effect on BBR severity is uncertain as statistical comparisons of means among treatments were not performed in the reported study (Reglinski *et al.*, 2010). Other proven plant defence inducers include salicylic acid and jasmonic acid (Elmer and Reglinski, 2006). While it has been shown that these chemicals can induce plant defence responses, studies demonstrating that these chemicals reduced BBR severity in grapes were not supported by statistical mean comparisons between treatments (Elmer & Reglinski, 2006, Reglinski *et al.*, 2010).

A number of factors are limiting the uptake of these new products in preference to fungicides. Firstly, the control provided by BCAs and alternative products has not been shown to match or exceed that provided by fungicides where direct comparisons for particular spray timings have been made. Additionally, the cost of commercialising a product can be prohibitive. Agrichemical companies may not be willing to invest in commercialising non-fungicidal products until traditional products are withdrawn or MRLs make fungicide use non-viable (Elmer & Reglinski, 2006).

2.3.6.3. Cultural techniques

Fungicides, BCAs and alternative products are not the only options grape growers have at their disposal for managing BBR. Various cultural techniques have been shown to provide effective control of the disease. Grape growers need to consider these techniques from the time the vines are planted. Trellis systems (Section 2.1.6) can reduce BBR severity by altering the canopy microclimate so that it is less conducive to infection by *B. cinerea* (Savage & Sall, 1984, Gubler *et al.*, 1987). In regions with a high risk of BBR, a trellis system that provides a more open canopy will increase air flow and may assist spray penetration (Stapleton *et al.*, 1995). Canopy density can also be controlled each season by methods such as leaf removal and shoot thinning, which can be carried out by hand or mechanically. The removal of leaves to open up the canopy and increase fruit exposure, has been shown to reduce either BBR incidence or severity in a number of trials (Gubler *et al.*, 1987, Zoecklein *et al.*, 1992, English *et al.*, 1993, Agnew *et al.*,

2004, Valdés-Gómez *et al.*, 2008, Hill *et al.*, 2011). Leaf removal has even been shown to result in BBR severity at harvest equal to a full fungicide regime (Beresford *et al.*, 2008). Shoot thinning, the removal of shoots early in the season to produce a thinner canopy and lower yield through either manual, mechanical or chemical means, is also believed to reduce BBR severity; however, there is little evidence to support this theory with some studies showing no such effect (Tardaguila *et al.*, 2008, Spring & Viret, 2009, Martinson, 2010). As a last resort, harvest times can be brought forward or more selective harvesting (harvesting from individual rows or even individual vines at different times) can be used. This can mean sacrificing a higher °Brix or other favourable juice characters in order to reduce overall crop loss.

Cultural techniques are labour intensive and can be a significant expense for grape growers. Leaf thinning carried out by machine can be as low as NZD$100 per hectare. Hand thinning, if required, can increase costs to more than NZD$400 per hectare (Hoksbergen, 2011). An increasingly popular and cost effective method of leaf thinning is to release sheep onto a vineyard (Emms, 2010). This method is not suitable for vineyard blocks where young vines are present and requires close monitoring to ensure the canopy is not excessively thinned.

2.4. Summary

This literature review shows that substantial scientific knowledge is available about the biology of *B. cinerea* in grapevines and the bunch rot disease that it causes, but gaps in our understanding of this pathosystem remain.

Infection by *B. cinerea* has been investigated, but the focus has primarily been on the flowering stage. Later phenological stages have not been investigated in as much detail and may be equally important in terms of infection, establishment of latent infection and the resulting BBR severity at harvest. The disease has been studied throughout the season and during the latent period, but the biology of the pathogen at the flower or berry level during this time is still unclear. Little is known about the *in planta* growth of *B. cinerea* at different phenological stages and during latency. The importance of BBR for grape growers and wine makers has also been described above. However, questions remain regarding the quantification of the disease, which brings into question the relevance of strict disease thresholds imposed by wineries.

The research objectives outlined in the introduction to this thesis (Chapter 1) are based on the knowledge gaps identified in this review. The subsequent chapters address these objectives in order to further improve our overall understanding of BBR in wine grapes.

3

Use of nitrate non-utilising (*nit*) mutants to determine phenological stages at which *Botrytis cinerea* infection establishes in wine grapes

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Accepted by *Plant Pathology* on March 7th, 2014.

3.1. Abstract

Botrytis bunch rot (BBR), caused by *Botrytis cinerea* Pers., degrades wine grapes during ripening, even though infection can occur as early as flowering. Effective BBR management requires knowledge of whether some stages of fruit development are more important than others in relation to infection and harvest BBR severity. Bunches of *Vitis vinifera* L. Sauvignon blanc and/or Pinot noir were inoculated in two vineyard trials and one glasshouse trial with nitrate non-utilising (*nit*) mutant strains at three phenological stages: early flowering, pre-bunch closure (PBC) and veraison. Isolates recovered from asymptomatic berries at veraison and from symptomatic bunches at harvest were screened to measure the incidence of the *nit* strains used in the inoculations. It was found that latent infections, which resulted in BBR at harvest, could become established at all three phenological stages and no single stage was associated with greater latent incidence or harvest severity than any other stage. It was concluded that a proportion of BBR at harvest resulted from the expression of latent infections that had accumulated throughout the season. However, the time between infection and BBR symptom expression in near-ripe grape berries was sufficiently short for polycyclic secondary infection to also contribute to epidemic development.

3.2. Introduction

Botrytis cinerea Pers. is a necrotrophic fungus that causes botrytis bunch rot (BBR) in *Vitis vinifera* L. grapevines. BBR results in seasonal variation in grape yield and quality and is one of the most economically important diseases of wine grapes (Nair & Hill, 1992; Loschiavo *et al.*, 2010). The use of fungicides for BBR management is becoming more restricted as many countries aim to produce wines with no detectable residues of a wide range of chemicals (New Zealand Winegrowers, 2013). Fungicide applications need to be timed strategically both to satisfy these needs and to achieve acceptable disease control. Knowledge of the time during grapevine fruit development that infection occurs is needed to guide fungicide timing.

Botrytis cinerea can be found in all floral parts during the early stages of flowering (Nelson & Amerine, 1956; Keller *et al.*, 2003; Viret *et al.*, 2004), demonstrating that floral infection can occur (McClellan & Hewitt, 1973). Most damage to the grape crop by BBR generally occurs during the pre-harvest period when susceptibility to infection increases as the grape berries ripen (Hill *et al.*, 1981). While infection by *B. cinerea* can

occur at any time from flowering, when the first caps fall, until after veraison, when the berries are ripening (Elmer & Michailides, 2004), the predominant sites and timing of infection in relation to flower and fruit development are not well understood.

The time between *B. cinerea* infection and sporulation is known as the latent period. Infections that persist in the host for an extended period without producing symptoms are called latent infections. Although the concept of latent infection by *B. cinerea* in grapes was first described in the 1970s (McClellan & Hewitt, 1973), its importance in BBR development is still largely unclear. Nair *et al.* (1995) found a strong logarithmic relationship between the incidence of *B. cinerea* infection in flowers and the incidence of berry infection at harvest, although it is not known how many vineyards, varieties or years were included in that study. Subsequently, Beresford and Hill (2008) found a significant but weak logarithmic relationship between incidence of latent infection in berries at pre-bunch closure (PBC) and BBR severity at harvest, using data from multiple vineyards in cool-climate regions in Australia and New Zealand.

It is not fully understood whether BBR epidemics that develop during grape ripening are predominantly the result of the expression of accumulated latent infections or polycyclic secondary infection (Zadoks & Schein, 1979) by *B. cinerea* spores produced within diseased bunches. A better understanding is needed of when latent infections establish under field conditions, the extent to which they persist through to harvest and their contribution to BBR symptom expression.

The overnight freezing and incubation technique (ONFIT), originally used for the detection of *Monilinia fructicola* in stone fruit (Michailides *et al.*, 2000), can be used to quantify latent infection in grape berries. This method incorporates freezing to degrade berry tissue, and presumably antifungal compounds (Pezet *et al.*, 2003), allowing the fungus to grow more easily. Surface sterilisation removes contaminants on the skin surface and increases the likelihood that growth of *B. cinerea* observed following incubation is attributable to latent infection. The inoculation of grape berries with a marked strain of *B. cinerea* that could subsequently be recovered and identified would allow the role of latent infections to be investigated.

Nitrate non-utilising (*nit*) mutants, which are fungal strains that are unable to utilise nitrate (NO_3) as a nitrogen source, have been produced for a number of fungi (Correll *et al.*, 1987; Brooker *et al.*, 1991; Beever & Parkes, 2007). They are useful as marked strains because other available nitrogen sources in the field mean they have similar fitness to naturally occurring wild-type strains. They can be used in field inoculations and

subsequently identified when re-isolated from infected host tissues. These mutants are produced on minimal media (MM) lacking nitrogen but amended with chlorate (ClO_3). As chlorate is an analogue of nitrate, wild-type strains convert it to chlorine, which is toxic to the fungus, whereas *nit* mutants do not metabolise chlorate and survive (Beever & Parkes, 2003).

The *nit1* mutant (Weeds *et al.*, 1998) putatively lacks the nitrate-reductase gene and the *nitM* mutant (Beever & Parkes, 2003) putatively lacks the molybdenum cofactor gene. Each *nit* pair belongs to the same vegetative compatibility group (VCG; Beever & Parkes, 2003), allowing the distinction of different *nit* pairs. When the *nit1* and *nitM* strain from the same VCG are grown together on the MM amended with nitrate (Beever & Parkes, 2003), anastomosis occurs and the mutated genes are replaced in the resulting fungal hyphae through complementation (Brooker *et al.*, 1991). This results in wild-type growth and confirmation of the strain phenotype.

The aim of this study was to use field and glasshouse inoculation with *nit* mutant strains of *B. cinerea* to investigate whether the amount of infection that established in grape bunches varied with grapevine phenological stage. Both latent infection and BBR severity at harvest were examined in either non-inoculated bunches or bunches inoculated with *nit* mutants. Evidence was also sought on the relative importance of accumulated latent infection and polycyclic secondary infection in BBR epidemiology.

3.3. Materials and methods

3.3.1. Nitrate non-utilising (*nit*) mutants

Three complementary *nit1*/*nitM* pairs, N1-1/N1-M, N2-1/N2-M and/or N3-1/N3-M, were used for inoculations in the field or glasshouse. All pairs were generated from *B. cinerea* isolates collected by Dr Ross Beever (Landcare Research, Auckland, New Zealand). The N1 pair was generated from an isolate collected in 2009 from *Vitis vinifera* Sauvignon blanc grown in a vineyard in Auckland, NZ. The N2 pair was generated from an isolate collected from *Actinidia deliciosa* 'Hayward' collected in 1982 from an orchard in Auckland, NZ. The N3 pair was generated from an isolate collected in 1985 from *Vitis vinifera* Müller-Thurgau grown in a vineyard in Blenheim, NZ. The N2 and N3 pairs were obtained from the International Collection of Micro-organisms from Plants (ICMP)

held at Landcare Research. ICMP accession numbers for the N2 pair are: N2-1 = 14122, N2-M = 14123 and for the N3 pair are: N3-1 = 14124, N3-M = 14125. Strains were selected for each trial based on their abundance of sporulation on malt extract agar (MEA) and their performance in previous trials.

3.3.2. Field trials

The effect of *B. cinerea* inoculation at different grapevine phenological stages on severity of BBR at harvest was investigated in two field trials: one during 2010/11 and the other during 2012/13 at a research vineyard in Auckland, NZ (-37.2038, 174.8636). A 25-year-old vineyard of Sauvignon blanc vines planted at 1.5 m spacing and 2 m between rows was used in 2010/11. The 2012/13 trial used 2-year-old vines planted at 1 m spacing and 2 m between rows with 25 vines of Sauvignon blanc alternating with 25 vines of *V. vinifera* Pinot noir within each row.

In 2010/11, two treatments were tested: non-inoculation (Treatment 1) or inoculation (Treatment 2) with a different *nit1* strain at each of the three phenological stages (Table 3.1). Five vine rows were used as blocks in a randomized block design, each separated

Table 3.1. Grapevine phenological stage, inoculation date and strain of *Botrytis cinerea* used for each treatment for both field trials and the glasshouse trial. Modified Eichhorn-Lorenz (E-L; Coombe, 1995) stages used to identify phenological stages were: flowering = E-L 19 âĂŞ E-L 26, pre-bunch closure (PBC) = E-L 32 and veraison = E-L 35.

Season	Trial type	Treatment	Phenological stage	Date	*Nit1* strain
2010/11	Field	1	Non-inoculated	-	-
		2	Flowering	09 December 2010	N1-1
			PBC	18 January 2011	N2-1
			Veraison	25 February 2011	N3-1
2011/12	Glasshouse	1	Non-inoculated	-	-
		2	Flowering	09 December 2011	N2-1, N3-1 or both
		3	PBC	24 January 2011	N2-1, N3-1 or both
		4	Veraison	20 February 2012	N2-1, N3-1 or both
2012/13	Field	1	Non-inoculated	-	-
		2	Flowering	11 December 2012	N2-1
		3	PBC	25 January 2013	N2-1
		4	Veraison	24 February 2013	N2-1

from the next by one non-inoculated buffer row. Each block contained two plots, one per treatment, separated by two non-inoculated buffer vines. Plots consisted of five or six vines. Prior to flowering, 40 inflorescences per plot were tagged arbitrarily. All 40 inflorescences or bunches were inoculated at the designated phenological stage with the designated *B. cinerea* strain (Treatment 2).

In 2012/13, four treatments were applied to each vine variety: a non-inoculated control treatment and three treatments with vines inoculated with N2-1 at one of three phenological stages (Table 3.1). Four vine rows per variety were used as blocks in a randomised block design, each separated from the next by one non-inoculated buffer row. Each block consisted of four plots, one per treatment, separated by two non-inoculated buffer vines. Each plot encompassed two rows so as to include both varieties. Every bunch in each plot was inoculated at the designated phenological stage.

3.3.3. Glasshouse trial

In 2011/12, a glasshouse trial was conducted at the Pukekohe Research Centre (Auckland, NZ). Flowering grapevine cuttings (Mullins cuttings) were established using a method modified from that described by Mullins and Rajasekaran (1981). Lengths of 1-year-old cane wood with 4–6 nodes were collected on 29 June 2011 from Sauvignon blanc and

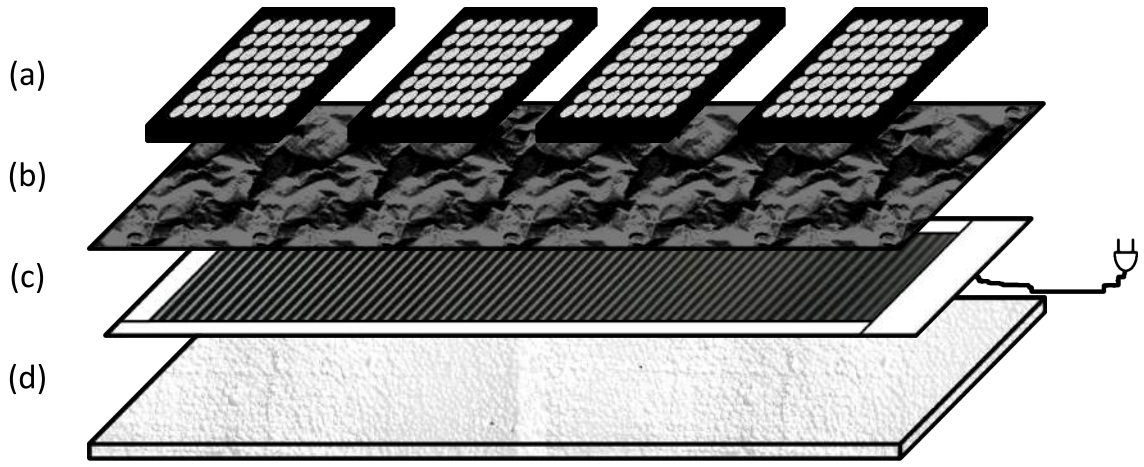

Figure 3.1. System for the propagation of one-node cuttings modified from Mullins and Rajasekaran (1981). (a) propagation trays filled with perlite; (b) polyurethane sheet; (c) heating pad; (d) 10 mm thick polystyrene.

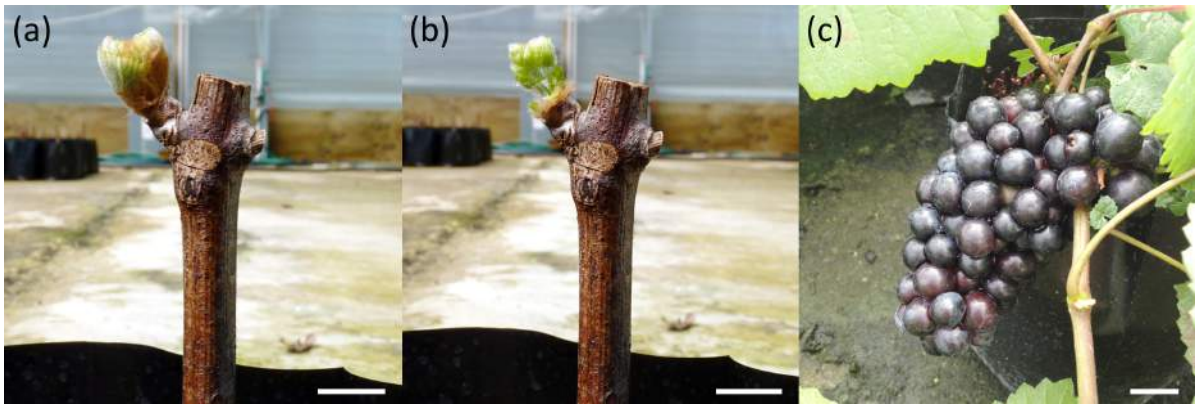

Figure 3.2. *Vitis vinifera* Pinot noir cutting before (a) and after (b) the removal of leaves surrounding the developing inflorescence, and with a fully developed bunch prior to harvest (c). Scale bars = 1 cm.

Pinot noir vines in a commercial vineyard in Waimauku, Auckland (-36.7433, 174.5064). The canes were stored in a cool room at 4°C until required. Single nodes were cut from the canes and each cutting was treated with IBAdex rooting hormone (Egmont Commercial Ltd, Christchurch, NZ) immediately prior to transfer to a cell of a 60-cell propagation tray (Flight Plastic Packaging, Lower Hutt, Wellington, NZ) filled with perlite (Egmont Commercial Ltd, Christchurch, NZ). Each tray was placed onto flexible heating pads (Ag Pads; Nu-Klear, Auckland, NZ) (Figure 3.1). The air temperature was set to 4°C with the base of the perlite maintained at a temperature of approximately 25°C. Cuttings were watered as needed to keep the perlite moist by placing base trays under the propagation trays, pouring 750 ml water into the base trays and allowing the perlite to absorb the water through the drainage holes in the propagation tray for 60 min; this was repeated every 2 d. The propagation trays were transferred to a glasshouse once $>$ 1 cm root growth was evident on $>$ 50% of cuttings.

Cuttings exhibiting callus formation or root growth were transferred to PB 5 polythene planter bags (2.4 L; Egmont Commercial Ltd) filled with potting mix containing 9-month slow-release fertiliser (NZ Growing Media Ltd, Winton, NZ). Potted cuttings were placed on the glasshouse floor and monitored closely for bud burst. Leaves were removed using fine forceps as soon as they were accessible following bud burst, leaving only the developing inflorescences (Figure 3.2). Any newly developed leaves were removed until the shoot was approximately 4–6 cm long, at which point the shoot tip and any secondary inflorescences were excised leaving only the primary inflorescence in a terminal position; new leaves were not removed after this point. Potted cuttings that developed an inflorescence $>$ 2 cm long were arranged into three randomized blocks containing four

plots with 30 cuttings per variety per plot. Each plot in each block was randomly assigned to one of four treatments: a non-inoculated control and three treatments inoculated at different phenological stages (Table 3.1). Each block was inoculated with one of three strain combinations: N2-1, N3-1 or both N2-1 and N3-1. Multiple strains were used at each inoculation time in case there was variation among isolates in their pathogenicity and fitness. Cuttings were sprayed with Lime Sulphur (Yates, Auckland, NZ) weekly between bud burst and flowering to control powdery mildew.

A weather station was placed in the glasshouse incorporating an HMP45A temperature/relative humidity probe (Vaisala Oyj, Helsinki, Finland) and a 237 leaf wetness sensor (Campbell Scientific, Inc., Logan, UT, USA) connected to a CR800 data logger (Campbell Scientific, Inc., Logan, UT, USA). Overhead sprinklers were used to water the cuttings and overhead misters were activated for 60 s in the morning and evening to ensure sufficient surface wetness throughout the trial to promote infection by *B. cinerea*.

3.3.4. Inoculation

Dry spore inoculations were used in this study, rather than spore suspensions, to better simulate natural infection. Each isolate of *B. cinerea* was grown on MEA (Oxoid Ltd, Basingstoke, Hampshire, UK) under fluorescent lights with a 12-h day length at room temperature for 10 d. Conidia were collected using a glass cyclone spore collector (Teng & Close, 1977) connected to a water aspirator. Dry conidia and/or sterile talcum powder were transferred to the main chamber of a glass microsprayer (Agar Scientific Ltd, Stansted, Essex, UK); weights of dry conidia varied from 0.2 to 2.8 g for each inoculation. Inflorescences inoculated at flowering were inserted inside the glass outlet tube and the rubber aspirator was squeezed once, producing a visible puff of spores. For inoculations at PBC and veraison, bunches were placed inside wind shields made from polyethylene terephthalate (PET) soft drink bottles attached to the glass outlet to maximise spore deposit on the target. Bunches remained inside the wind shields for 5 s after inoculation to allow conidia to settle on the grape berry surfaces. For the glasshouse trial, overhead water misters were activated for 30 s following each inoculation to create surface moisture on grape tissues.

After each set of inoculations, spores were applied with a single puff onto the surface of water agar (1.5% w/w) in three Petri plates which were incubated for 36 h at 15–20°C under fluorescent lights with a 12-h photoperiod. Average spore density (spores/mm^2)

and average germination rate (%) for each set of inoculations was determined using 100 x magnification and observing all spores in a 2.5-mm diameter field of view.

3.3.5. Quantification of latent infection

For the field trials, bunches were collected from each plot one week after veraison for quantifying latent infection by *B. cinerea*: 10 bunches per plot in 2011 and three bunches per plot for the Sauvignon blanc vines only in 2013. For the glasshouse trial (2011/12), three Sauvignon blanc bunches were collected from each plot 1 week after each inoculation. The overnight freezing and incubation technique (ONFIT) was carried out following collection.

Bunches were placed in individual plastic bags and placed in a freezer at -20°C. After 24–48 h, 20 berries without BBR symptoms were selected arbitrarily from each bunch and surface sterilised in 70% ethanol for 10 s followed by immersion in 2% sodium hypochlorite (NaOCl) for 1 min. The berries were placed onto a paper towel to absorb excess NaOCl. A paper towel, soaked in Botran 75 WP (750 mg/L dicloran (DuPont (New Zealand) Ltd, Auckland, NZ) to reduce contamination by *Rhizopus stolonifer*, was placed into a plastic container (175 x 120 x 40 mm). To prevent the berries rolling, a piece of polyvinyl chloride (PVC) mesh with a 1 cm^2 grid, commonly used as a non-slip underlay for carpet rugs, was placed on the paper towel and berries sampled from one bunch were placed into the cells created by the mesh. A lid was placed loosely over the plastic container to allow some gas exchange and the container was placed on a laboratory bench at 15–20°C with exposure to daylight, but not direct sunlight. After 10 d the percentage of berries with sporulating *B. cinerea* was recorded and this was interpreted as the percentage incidence of latent infection. Berries were examined using a stereo microscope for the presence of *B. cinerea* conidiophores where confirmation of fungal species was required.

3.3.6. Soluble solids

Thirty berries were collected arbitrarily from each variety in each replicate in each trial and placed in a plastic bag. The berries were crushed by hand and the juice was pipetted onto the glass reading surface of a Pal-1 digital refractometer (Atago Co., Ltd; Tokyo,

Japan) and the soluble solids concentration (°Brix) was recorded. Mean °Brix for each variety in each trial was calculated.

3.3.7. Disease assessment

BBR severity at harvest (E-L 38) was measured visually by estimating the percentage of tissue exhibiting BBR symptoms when viewed from one side of the bunch (Hill *et al.*, 2010). If there was doubt about the cause of the symptoms, then the presence of *B. cinerea* sporulation was used to confirm BBR.

For the field trials, BBR severity was recorded for all tagged bunches remaining on the vine at each assessment date. The 2010/11 field trial was assessed on 04 April in 2011. For the 2012/13 field trial, all bunches per plot were assessed on 05, 15 and 20 March 2013, with the latter date approximating the commercial harvest date (Table 3.2). In the glasshouse trial, BBR severity was recorded for all Mullins cuttings with an intact grape bunch on 16 March 2012.

3.3.8. Isolate recovery and *nit* mutant screening

B. cinerea strains were isolated from the berries incubated to reveal latent infection and from infected berries on field vines and Mullins cuttings at harvest. A sterile wooden toothpick was used to transfer conidia from the infected berry to MEA in a Petri plate. The MEA was amended with 2.5 ppm dicloran and 100 ppm streptomycin. Up to three samples of conidia per incubation tray, each from individual berries, were transferred to MEA depending on the number of infected berries present. One isolate was taken from each infected bunch at harvest. After 7 d incubation as described previously, conidial suspensions in sterile H_2O were prepared from each culture and stored at -20°C for up to 90 d.

To identify isolate strain type, conidial suspensions were thawed on ice prior to overlaying 3 μl of conidial suspension and 3 μl of a relevant *nitM* strain on minimal media (MM; Beever & Parkes, 2003) amended with 10 mM potassium nitrate (KNO_3) and 0.05% Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA) (Figure 3.3). The *nitM* strains used were N1-M, N2-M and N3-M according to the *nit1* strains used in a trial. *NitM* strains were only used for screening where the complementary *nit1* strain was

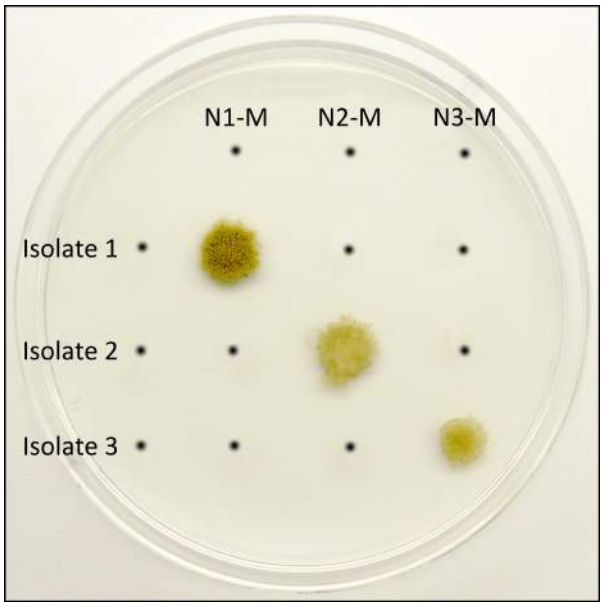

Figure 3.3 Minimal media used for screening *Botrytis cinerea* isolates where *NitM* strains (N1-M, N2-M and N3-M) were plated in each column. An isolate of unknown strain type was plated across one row. Wild-type mycelial growth occurred when a *nit1* strain was in the same position as a complementary *nitM* strain, identifying the strain as either N1-1, N2-1 or N3-1. Isolates growing on multiple spots within the same row were considered wild-type strains. In this example, isolate 1 = N1-1, isolate 2 = N2-1 and isolate 3 = N3-1 and incubation time was 7 d.

used in a particular trial. Up to three isolates were screened per plate. The strain of each isolate was recorded after 7 d based on whether complementation had occurred with one of the *nitM* strains. If wild-type growth was observed without the need for complementation, the isolate was recorded as a wild-type strain.

3.3.9. Statistical analyses

All statistical analyses were done using GenStat 14^{th} Edition (VSN International, Hemel Hempstead, UK). Analysis of variance (ANOVA) combined with Fisher's least-significant difference (LSD) was used to compare means. Data were transformed prior to ANOVA to satisfy assumptions of homogeneity of variance. Data for germination rate, strain incidence and incidence of latent infection (%) were arcsine transformed:

$$f(x) = asin(\sqrt{x/100}) \times 180\pi \tag{3.1}$$

where x = the variable to be transformed. Data for BBR severity (%) were transformed using the following equation:

$$f(x) = ln\left(\frac{x + 0.1}{100.1 - x}\right) \tag{3.2}$$

where x = BBR severity (Beresford *et al.*, 2006).

Analysis of covariance (ANCOVA) was used to analyse the incidence of latent infection (%) and BBR severity (%) at harvest for the glasshouse trial and 2012/13 field trial. The proportion of isolates identified as one of the *nit1* mutant strains used for inoculations was calculated for each experimental plot and this value was used as the covariate in ANCOVA. The goal was to obtain a more reliable estimate of incidence or severity associated with infection resulting from inoculations and to eliminate wild-type infections from the analyses. The incidence of latent infection and BBR severity data referred to herein are the fitted means from these analyses.

Data from the glasshouse trial and the 2012/13 field trial were combined for ANCOVA as they had the same treatment structure. Trial and variety were used as nested blocking effects, in that order. *Nit1* strain (N2-1, N3-1 or both N2-1 and N3-1) was used as an additional nested blocking effect for data from the glasshouse trial to account for potential variability in strain pathogenicity.

Data for mean BBR severity for the 2012/13 field trial collected at each assessment date were combined for Pinot noir and Sauvignon blanc varieties for ANOVA with variety as a blocking effect.

3.4. Results

3.4.1. Latent infection

In the 2010/11 field trial, none of the 21 *B. cinerea* isolates recovered by ONFIT at veraison from non-inoculated bunches (Treatment 1) were *nit1* strains (Table 3.2). Of the 105 isolates obtained from berries inoculated with *nit1* strains (Treatment 2), 99 were the *nit1* strains used in the inoculations and six were wild-type strains. A significantly ($P < 0.05$) greater percentage of isolates was identified as strains N1-1 and N2-1, applied at flowering and PBC respectively, than those identified as strain N3-1, applied at veraison (Figure 3.4a). In Treatment 2, two different strains were isolated from the same bunch

Table 3.2. Sampling for *Botrytis cinerea* at veraison and harvest in two field trials and one glasshouse trial investigating recovery of *B. cinerea nit1* strains inoculated onto grape bunches at three growth stages. Number of bunches sampled and number of isolates recovered are shown for latent infection at veraison, when the overnight freezing and incubation technique (ONFIT) was used. At harvest, the number of bunches assessed for botrytis bunch rot (BBR) severity, the number of bunches sampled for isolation of *B. cinerea* and the corresponding number of isolates obtained are shown. The number of *nit1* isolates includes isolates that represented at least one of the three *nit1* strains used in inoculations (Table 3.1).

				Veraison			Harvest			
Season	Trial type	Variety	Treatment[1]	Bunches sampled	Isolates (*nit1*)	Isolates (total)	Bunches assessed	Bunches sampled	Isolates (*nit1*)	Isolates (total)
2010/11	Field	Sauvignon blanc	1	50	0	21	-	75	0	61
			2	50	99	105	-	75	29	59
2011/12	Glasshouse	Pinot noir	1	92	15	29	24	9	0	3
			2	92	26	28	23	9	2	4
			3	92	21	29	22	9	5	6
			4	92	31	37	21	9	0	3
		Sauvignon blanc	1	-	-	-	13	9	2	2
			2	-	-	-	9	9	3	3
			3	-	-	-	9	9	3	3
			4	-	-	-	11	9	3	4
2012/13	Field	Pinot noir	1	-	-	-	38	4	5	16
			2	-	-	-	38	6	14	17
			3	-	-	-	53	9	19	25
			4	-	-	-	44	12	18	19
		Sauvignon blanc	1	12	4	4	68	16	1	4
			2	12	21	21	67	17	6	6
			3	12	8	8	67	25	6	9
			4	12	13	13	77	19	9	12

[1] Refer to Table 3.1.
[2] Treatments also subjected to ONFIT at flowering and pre-bunch closure (data not shown).

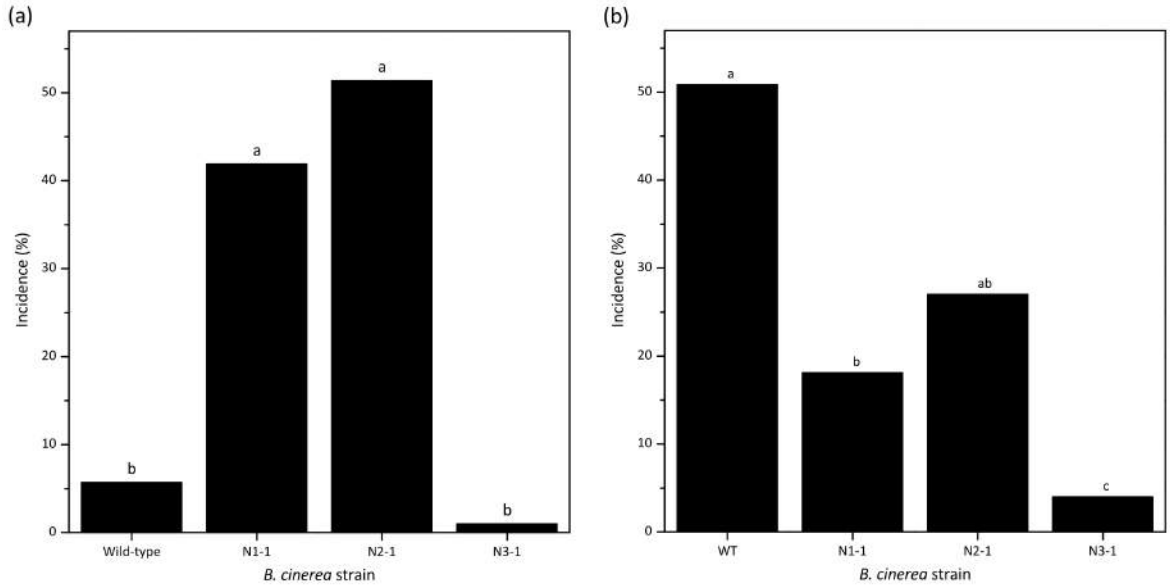

Figure 3.4. Incidence of *nit1* mutant (N1-1, N2-1 and N3-1) or wild-type *Botrytis cinerea* strains isolated from inoculated (Treatment 2) Sauvignon blanc bunches in the 2010/11 field trial obtained at (a), veraison using the overnight freezing and incubation technique ($n = 105$) and (b) at harvest from botrytis bunch rot symptoms ($n = 59$). Statistical analyses were performed using arcsine transformed data. Means with the same letter are not significantly different ($P < 0.05$).

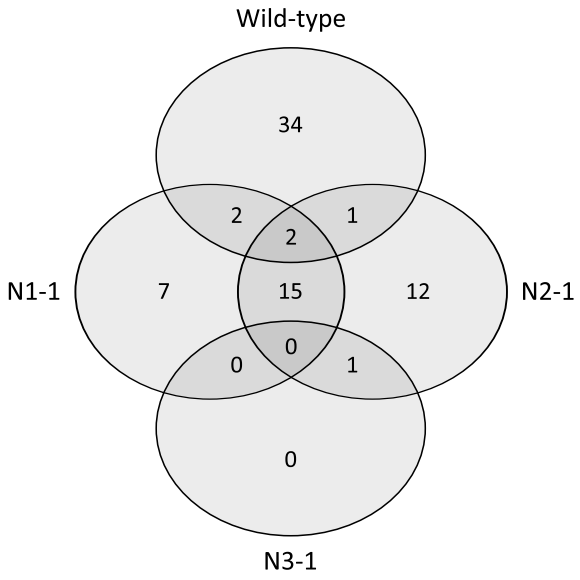

Figure 3.5 Number of Sauvignon blanc bunches collected at veraison from Treatment 2 in the 2010/11 field trial with one or more isolates representing the N1-1, N2-1, N3-1 and/or wild-type strain combination. Bunches were inoculated at flowering (N1-1), pre-bunch closure (N2-1) and veraison (N3-1).

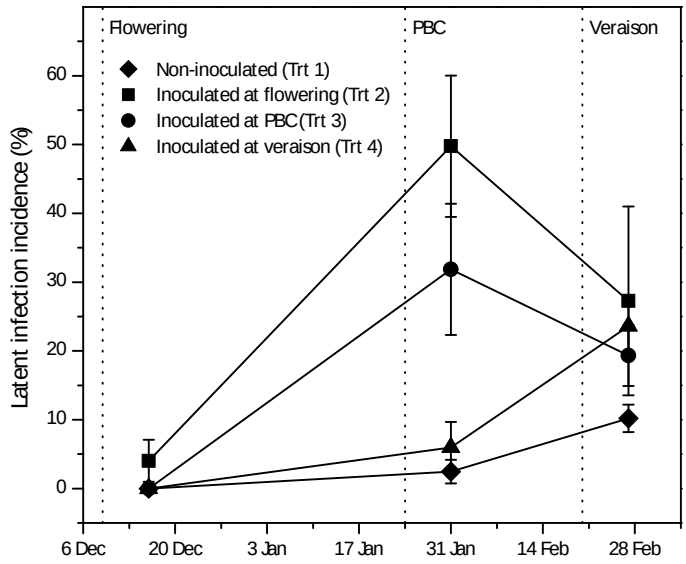

Figure 3.6 Incidence of *Botrytis cinerea* strains N2-1 and N3-1 recovered using the overnight freezing and incubation technique (ONFIT) from Pinot noir bunches 1 week after these strains were inoculated (dotted lines) at flowering (Treatment 2), pre-bunch closure (PBC; Treatment 3) or veraison (Treatment 4) in the 2011/12 glasshouse trial. Error bars represent standard error (d.f. = 2).

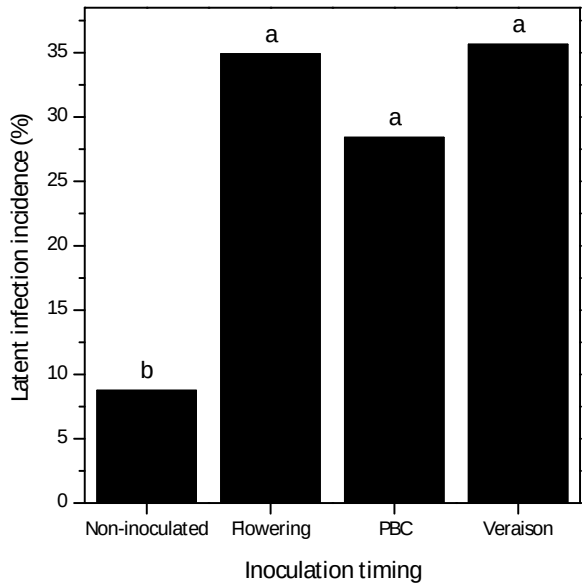

Figure 3.7 Mean percentage of berries infected with *Botrytis cinerea,* adjusted by analysis of covariance using the percentage of isolates identified as the N2-1 or N3-1 strains as a covariate, following the overnight freezing and incubation technique (ONFIT) at veraison. Means are averages for all plots in each treatment in the 2011/12 glasshouse trial and 2012/13 field trial (Pinot noir and Sauvignon blanc vines, respectively). Statistical analyses were performed on transformed data. Means with the same letter are not significantly different ($P < 0.05$).

for 19 bunches sampled and three different strains were isolated from the same bunch for two bunches sampled (Figure 3.5).

In the glasshouse trial, the percentage of isolates recovered that were the N2-1 or N3-1 strain applied at flowering, PBC or veraison (Treatments 2, 3 and 4, respectively) was greater following inoculation of the respective treatment than before inoculation (Figure 3.6). This result indicated that latent infection was present at all growth stages sampled and that inoculation with *B. cinerea* substantially increased the amount of latent infection. *Nit1* strains applied at all three growth stages were recovered after veraison and at harvest (Table 3.2), indicating that latent infections that established as

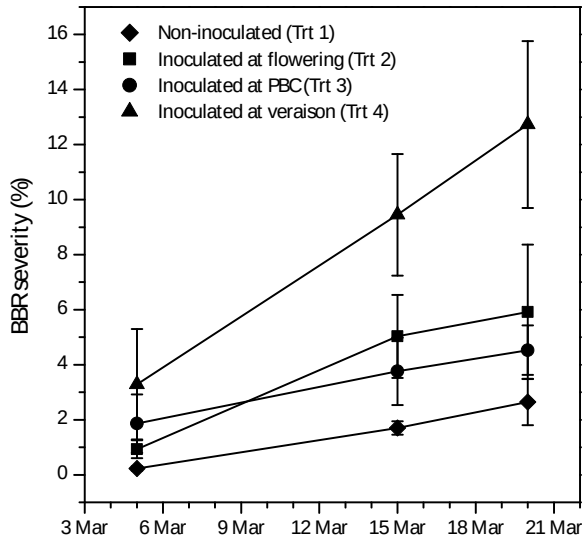

Figure 3.8 Mean botrytis bunch rot (BBR) severity averaged for Pinot noir and Sauvignon blanc for all treatments in the 2012/13 field trial. Error bars represent standard error (d.f. = 7).

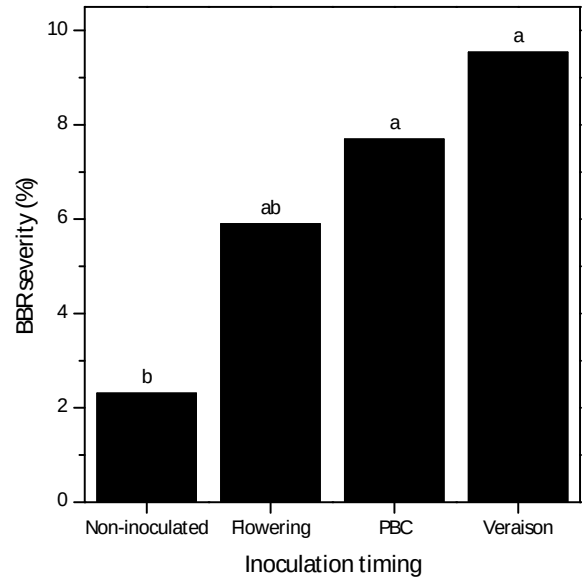

Figure 3.9 Mean botrytis bunch rot (BBR) severity at harvest, adjusted by analysis of covariance using the percentage of isolates collected at harvest identified as the N2-1 strain as a covariate, for both Pinot noir and Sauvignon blanc varieties in both the glasshouse trial and 2012/13 field trial. Statistical analyses were performed on transformed data. Means with the same letter are not significantly different ($P < 0.05$).

early as flowering were able to persist throughout the season. There was greater recovery of the *nit1* strains from Treatment 2 after PBC than after the flowering stage at which Treatment 2 was inoculated. However, the incidence of latent infections appeared to fluctuate in cuttings inoculated at flowering and PBC, with a drop of 10–20% between the second and third assay for latent infection (Figure 3.6). A small incidence (6%) of *nit1* strains was obtained from Treatment 4 at PBC, even though that treatment was not inoculated until veraison. This result, along with isolation of the *nit1* strains from Treatment 1 (non-inoculated control) after the PBC and veraison inoculations, indicated a small amount of cross-contamination of strains between experimental plots. Wild-type strains were isolated from all treatments at all growth stages (Table 3.2).

When veraison latent infection incidence data were combined for the 2011/12 glasshouse trial and 2012/13 field trial, it was evident that inoculation caused a significant ($P < 0.05$) increase in the percentage of berries with N2-1 or N3-1 strains recovered compared with the non-inoculated control. However, there was no significant ($P < 0.05$) difference between the three inoculation times (Figure 3.7), indicating that latent infection can establish at any of these growth stages.

3.4.2. BBR at harvest

Mean soluble solids concentration at harvest (E-L 38) in the 2010/11 field trial was 19.8 ^{o}Brix. In the glasshouse trial it was 16.5 and 18.9 ^{o}Brix for Pinot noir and Sauvignon blanc, respectively, and in the 2012/13 field trial it was 17.2 and 22.7 ^{o}Brix for Pinot noir and Sauvignon blanc, respectively.

In the 2010/11 field trial, all 61 *B. cinerea* isolates collected at harvest from non-inoculated bunches were wild-type strains (Table 3.2), indicating that cross-contamination from inoculated *nit1* strains had not occurred. Isolates collected from Treatment 2 included 30 wild-type strains and 29 *nit1* strains representing all three *nit1* strains used in inoculations. The proportion of wild-type strains at harvest was much greater than was found for latent infection at veraison, indicating that substantial infection from background sources of inoculum had occurred after veraison. Strains N1-1 and N2-1 applied at flowering and PBC represented a significantly ($P < 0.05$) greater percentage of isolates than strain N3-1 applied at veraison (Figure 3.4b). BBR severity at harvest was not assessed as the grape bunches were affected by other biotic stresses, including powdery mildew and sour rot.

In the 2012/13 field trial, mean BBR severity for Treatment 4 (inoculated at veraison) was significantly ($P < 0.05$) greater than Treatment 1 (non-inoculated control) on all assessment dates and significantly ($P < 0.05$) greater than Treatments 2 and 3 (inoculated at flowering and PBC, respectively) on 20 March 2013 (Figure 3.8).

When BBR severity data were combined for both the glasshouse trial and 2012/13 field trial, there were no significant differences ($P < 0.05$) among inoculation treatment means (Figure 3.9). Mean BBR severity was significantly higher ($P < 0.05$) in Treatments 3 and 4 (inoculated at PBC and veraison, respectively) than the percentage for the non-inoculated control. Mean BBR severity for Treatment 2 (inoculated at flowering) was not significantly different ($P > 0.05$) from any other treatment.

3.5. Discussion

Primary infection by *Botrytis cinerea* was established successfully within 1 week of inoculation of dry conidia at each of the three phenological stages examined in this study: flowering, pre-bunch closure or veraison. It was demonstrated that these latent infections persisted through to harvest and resulted in BBR severity that was significantly higher than observed for non-inoculated bunches. There was no evidence to suggest BBR severity was higher as the result of infection at one particular phenological stage than another.

Flowering has previously been considered to be one of the more important growth stages for infection to occur because of the susceptibility of floral parts (Keller *et al.*, 2003), the potential for the establishment of latent infections (McClellan & Hewitt, 1973), and the correlation of the incidence of flower infection with BBR incidence at harvest (Nair *et al.*, 1995). Nair and Parker (1985) found an increase in the incidence of latent infections around PBC relative to other growth stages, but they stated that the infections were established at flowering because infected, undeveloped flowers were found inside bunches without considering that primary infection may have occurred at later growth stages. The majority of previous studies on latent *B. cinerea* infection, particularly those involving inoculations, have focussed primarily on the flowering or post-veraison period and have not simultaneously investigated intermediate growth stages for direct comparison (Nair *et al.*, 1995; Keller *et al.*, 2003; Pezet *et al.*, 2003). This study found that infection at the flowering stage does not necessarily lead to a higher incidence of latent infections than infection at other growth stages. A higher percentage of wild-type strains at harvest compared with veraison in the 2010/11 field trial suggests that infections continued to occur after veraison.

Latent infections may have continued to establish more than 1 week after the flowering inoculation, as evidenced by the observed increase in incidence between the flowering and PBC ONFIT assays in the 2011/12 glasshouse trial (Figure 6). BBR symptoms were first evident prior to the post-veraison ONFIT assay. Therefore, some of the latent infections present prior to veraison may have progressed to symptomatic infections. As the ONFIT is only carried out using asymptomatic berries, the incidence of latent infections might be underestimated if BBR symptoms are also present. For this reason, the relative differences between treatments at a given time are more important than the differences within treatments at different times.

The frequency of recovery of each *nit1* strain at harvest in 2011 varied significantly. It is not known whether this was because of the relative susceptibility of grape material at different growth stages or the relative pathogenicity or fitness of the strains used. Only 4% of the isolates collected throughout the glasshouse trial were the N3-1 strain, which suggests that the pathogenicity or fitness of this strain was lower than the N2-1 strain. If so, this result might explain the low incidence of the N3-1 strain at harvest in the 2010/11 field trial.

BBR severity normally increases exponentially between veraison and harvest (Beresford *et al.*, 2006) and this increase has been attributed to infected bunches becoming a source of inoculum for secondary infections, leading to a polycyclic epidemic (Elmer & Michailides, 2004). BBR symptoms in the field trials were not observed until after veraison regardless of inoculation timing, suggesting that latent infections do not develop into symptomatic infections until berries reach a suitable stage of maturity. Soluble solids concentration is one measure of berry maturity that reflects susceptibility to *B. cinerea* infection (Mundy & Beresford, 2007) and has been found to increase exponentially following veraison (Sadras & Moran, 2013). Therefore, it is possible that BBR is a monocyclic disease and that the exponential increase results from latent infections developing into BBR symptoms as berries mature without polycyclic secondary infection occurring. Infected floral parts and aborted berries trapped within bunches (bunch trash) may also contribute to an increase in severity as berries mature (Nair *et al.*, 1988). Bramley *et al.* (2011) investigated the spatial distribution of BBR severity and found that mean severity for a given vineyard block (2.4 ha) was heavily influenced by an increase in severity in particular areas within the vineyard, while severity in the rest of the block did not increase at the same rate. These findings support the hypothesis that polycyclic secondary infection may not be a necessary mechanism for increasing BBR severity over time.

Whether or not secondary infection contributes to the exponential increase in BBR severity near harvest would depend on the latent period in relation to the period between the first appearance of BBR and harvest. Vines inoculated at veraison in the 2012/2013 field trial developed 3.3% mean BBR severity 9 days after being inoculated (Figure 3.8). As this was 15 days prior to harvest, there was probably enough time for secondary infection and symptom development to occur. The *nit1* mutant (N2-1) isolates from non-inoculated bunches may have been the result of either secondary infection, or cross-contamination during inoculations. Air movement during inoculation meant it was difficult to contain spores to the bunch being inoculated. Cross-contamination is likely

to have occurred in the glasshouse trial as the plots were in very close proximity. Further investigation into the role of secondary infection in pre-harvest BBR development would need to address this issue either by more effective shielding during inoculations or by using an aqueous spore suspension.

The use of *nit* mutants in this study allowed inoculated strains to be identified and allowed the contribution of inoculation to BBR severity to be determined. However, there was a risk of contamination during the isolation and screening process due to the use of non-sterile field material. It was also possible that the *B. cinerea* strains isolated from bunches were not the only source of BBR symptoms, as multiple strains were isolated from single bunches in the 2010/11 field trial. Strains of *B. cinerea* with specific genetic markers could be detected using routine molecular analyses. This would eliminate the problem of contamination and allow whole bunches to be screened to identify all marked strains present. It could also be relatively simple to use multiple strains in each inoculation, to account for variability in strain pathogenicity and fitness, as the screening process would be no more difficult than with a single strain. Finding genetic markers that relate to currently known VCGs would allow *nit* mutant strains to be used for an additional screening method. However, the genetic basis for VCGs in filamentous fungi is not fully understood (Glass *et al.*, 2000). Asadollahi *et al.* (2013) identified substantial genetic diversity among *B. cinerea* isolates which could be used to identify new traceable strains. While previous studies have examined genetic diversity with a focus on population genetics (Fekete *et al.*, 2012; Asadollahi *et al.*, 2013), some of the isolates identified may be useful as traceable strains. The naturally occurring population would need to be assessed prior to conducting research in a particular region to ensure the marked strain(s) being used is not already present.

This study showed that infection of grape berries can occur at multiple phenological stages. If so, then BBR management actions need to be considered from flowering right through to harvest. As the use of fungicides becomes more restricted due to decreasing residue limits, the challenge lies in finding alternative management actions that are able to control and prevent BBR epidemics throughout the entire season.

3.6. Acknowledgements

This work was supported by the The New Zealand Institute for Plant & Food Research Limited and the Tasmanian Institute of Agriculture (TIA), University of Tasmania. We

thank Peter Wright and Glen Turner (Plant & Food Research, Pukekohe, Auckland, NZ) for their assistance is maintaining both the research vineyards and glasshouses. We also thank Karyn Hoksbergen and the late Dr Ross Beever (Landcare Research, Auckland, NZ) for all their assistance with *nit* mutant strains.

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Visualisation of grape flowers and berries inoculated with green fluorescent protein (GFP)-expressing *Botrytis cinerea*.

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Submitted to *Australasian Plant Pathology* on January 30th, 2014.

4.1. Abstract

Botrytis cinerea Pers. is a nectrotrophic pathogen that causes botrytis bunch rot (BBR) in grapes. While the infection of grape flowers and other host species by *B. cinerea* has been investigated using various microscopic techniques, infection and colonisation of grape berries does not appear to have been reported. This study investigated the infection of *Vitis vinifera* L. Crimson Seedless berries and *V. vinifera* Sauvignon blanc flowers and berries by a green fluorescent protein (GFP)-expressing strain of *B. cinerea* using fluorescence microscopy, confocal microscopy and brightfield light microscopy. Extensive mycelial growth was observed on Sauvignon blanc flowers; however, it was not clear whether penetration of the host tissue had occurred. Very little hyphal growth was observed on Sauvignon blanc berries collected and inoculated at pre-bunch closure (PBC). Staining samples with lactophenol cotton blue did not reveal substantially more hyphae on the berry surface than was seen with fluorescence microscopy. Growth in mature berries was similar to that seen previously in other host species. The majority of hyphae were highly branched and intercellular, growing between the cuticle and the epidermis, with some enlarged hyphae penetrating deeper into the berry tissue. Wounding was found to be important for the infection of Crimson Seedless berries and visibly less hyphal growth was observed on the surface of non-wounded, mature Sauvignon blanc berries compared with wounded berries.

4.2. Introduction

Botrytis cinerea Pers. is a nectrotrophic pathogen that causes botrytis bunch rot (BBR) in grapes (Nair and Hill 1992). The most common symptom of this disease on white grapes is darkened, rotting berries that become covered in grey, sporulating conidiophores. A second symptom is known as slip skin in which an infected berry appears asymptomatic until light pressure is applied at which point the skin slides easily from the pulp (Nelson 1956; Hewitt 1974). While BBR is one of the most economically important diseases in grapes (Scholefield and Morison 2009), many aspects of the infection process and latency are not well understood.

Elmer and Michailides (2004) reviewed mechanisms by which *B. cinerea* infection leads to pre- or post-harvest fruit disease of grapes and other fruit crops. They proposed five 'infection pathways' that relate to *B. cinerea* in grapes. However, these can be simplified into three general mechanisms by which mature berries can become infected:

1) infection of floral parts, including the stamens, style and pedicel, that leads to the establishment of latent infection, 2) infection of maturing berries by conidia or hyphae from infected floral parts, aborted berries or other grape tissues trapped in grape bunches, and 3) infection of maturing berries by conidia from an external inoculum source, either sporulating, infected bunches or other host species within the vineyard. The first two of these mechanisms are supported by observations of the location of *B. cinerea* mycelium growing from incubated infected berries (Holz et al. 2003; Keller et al. 2003; Sanzani et al. 2012) and bunch trash (Nair et al. 1988). While these studies identified the location of the pathogen in grape tissue, they did not necessarily address where the infections originated or whether or not latent infections remained quiescent.

Observing and understanding the infection process of *B. cinerea* requires various microscopy techniques. Viret et al. (2004) inoculated detached inflorescences with *B. cinerea* and obtained images of the infection process in grape flowers (Figure 4.1) using both transmission and scanning electron microscopy. Infections were found to be localised near the torus, providing further evidence for the incubation studies that found *B. cinerea* to grow predominantly from this part of the grape berry. Previous studies have used microscopy to investigate the infection processes and structures of *B. cinerea* in other host species (Clark and Lorbeer 1976; Backhouse and Willetts 1987; Fourie and Holz 1995; Zhang et al. 2010; Rheinlander et al. 2013). *Botrytis cinerea* enters the host via cuticle penetration or wounds, intercellular growth spreads between the cuticle and the epidermis, with enlarged hyphae penetrating deeper into host tissue, followed by death of host cells and hyphal invasion (Govrin and Levine 2000; van Kan 2005). No studies appear to have directly addressed the infection of grape berries.

Electron microscopy, as used by Viret et al. (2004), allows visual resolution of < 10 nm. However, plant and fungal material must be fixed, which prevents observation of processes occurring in living tissue; surveying large areas of tissue are also problematic at such a high magnification. Fluorescent microscopy combined with appropriate dyes can be used to observe fungi *in vivo* (Hickey et al. 2004); however, these dyes may not penetrate through to fungal tissue when growing *in planta* and visualization through cell-layers can be difficult. Confocal microscopy uses single-point illumination at the focal plane, eliminating out-of-focus blur resulting from full-field illumination used in light microscopy (Sheppard and Shotton 1997). A series of optical sections results in a 'z-stack', which comprises a series of images at different planes along the z-axis). These images can be combined to produce a single two-dimensional projection image of the volume observed or further processed to produce a three-dimensional image (Sheppard

and Shotton 1997). Under ideal conditions, fungal growth can be studied *in vivo* using fresh tissue without the potential damage to cell integrity that can be caused by fixing and staining the tissue (Lorang et al. 2001). Material can be visualised immediately and time-lapse microscopy applied to observe infection processes (Genre 2008).

Genetic modification of the fungus to express a cytoplasmic fluorescent protein, such as green-fluorescent protein (GFP; Shimomura et al. 1962; Lorang et al. 2001), provides an even greater opportunity for visualising fungi *in vivo* (Czymmek et al. 2002). This allows fluorescence to be introduced into the fungus without the use of dyes that may not be effective *in planta*. The two most common GFP variants used in the genetic transformation of filamentous fungi are synthetic GFP (sGFP; Chiu et al. 1996) and enhanced GFP (eGFP; Zhang et al. 1996). These variants have been successfully expressed in a number of phytopathogenic fungi in order to study them *in planta*, including *Magnaporthe grisea* in barley (Czymmek et al. 2002), *Sclerotinia sclerotiorum* in dry bean, canola, soybean and sunflower (de Silva et al. 2009), *Aspergillus carbonarius* in table grapes (Crespo-Sempere et al. 2011), *Phomopsis viticola* in the leaves and canes of Seyval wine grapes and *B. cinerea* in *Arabidopsis thaliana* (Leroch et al. 2011).

Working with GFP-expressing fungal strains can be challenging in both Australia and New Zealand because of strict guidelines for the handling of genetically modified organisms (GMOs). These restrict the research location and transport of the organisms beyond certified facilities. However, the guidelines are not entirely prohibitive and the benefits can outweigh the potential difficulties.

This study investigated the growth of *B. cinerea* in grape berries and flowers and the potential use for a GFP-expressing strain in microscopy studies. Infection was observed *in vivo* in mature *Vitis vinifera* L. Crimson Seedless table grapes imported from Chile, in which *B. cinerea* was observed without fixing, and in *V. vinifera* Sauvignon blanc wine grapes collected in an Auckland vineyard at flowering, pre-bunch closure and veraison, which were fixed prior to microscopy.

4.3. Materials and Methods

4.3.1. Grape material

Preliminary microscopy used mature *V. vinifera* Crimson Seedless table grapes imported from Chile, as these were available at the time the work was to be carried out. The

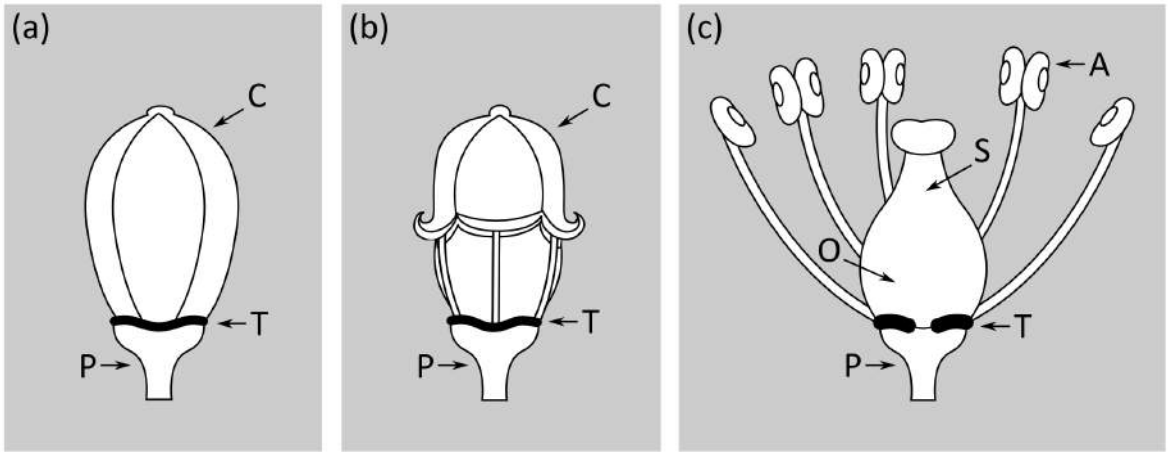

Figure 4.1. Diagram showing the morphology of a grape flower before (a), during (b) and after (c) capfall. A = anther, C = calyptra, O = ovary, P = pedicel, S = style and T = torus/receptacle. Illustration by Ben Galbraith.

grapes were stored at 4° C for up to 7 d until samples were inoculated, sectioned and visualised as living tissue, all within the same facility.

Subsequent microscopy used *V. vinifera* Sauvignon blanc wine grape samples collected from a research vineyard in Auckland, New Zealand (-37.2038, 174.8636) during the 2012/13 season. At each of three developmental stages, flowering (modified Eichorn-Lorenz stage 19; E-L 19; Coombe 1995), pre-bunch closure (PBC; E-L 32) and veraison (E-L 35), nine bunches were collected, placed in individual polyethylene bags, transported to the laboratory and inoculated on the same day. For logistical reasons, these samples were inoculated, fixed and sectioned within one facility and then transported to a second facility at which the confocal microscope was located.

4.3.2. GFP-expressing *Botrytis cinerea* and inoculation

The *B. cinerea* strain used in this study was a wild-type collected from a Sauvignon blanc vine in Auckland, New Zealand (-37.2038, 174.8636) and *Agrobacterium*-transformed with the pJK5-sGFP plasmid resulting in expression of the sGFP protein in the cytoplasm (Lee 2010). A suspension of conidia was prepared in sterile H_2O from cultures grown on malt extract agar (MEA) for 7 d. Spore concentration was measured using a haemocytometer and the suspension diluted to 1 x 10^5 spores/ml with sterile H_2O.

Prior to inoculation of Crimson Seedless grapes, a paper towel soaked in the fungicide Botran 75 WP (750mg/L dicloran; DuPont Ltd., Auckland, NZ) was placed into a sterile polyethylene container (130 mm^2 × 40 mm deep) to suppress growth of *Rhizopus* spp. An autoclaved piece of polyvinyl chloride (PVC) mesh (1 cm^2 grid), commonly used as a non-slip underlay for carpet rugs, was placed on the paper towel. Six berries were detached from the rachis with pedicels attached and placed into the cells created by the mesh to prevent rolling. Three of the berries were wounded prior to inoculation by pricking the middle of the berry cheek once with a flame-sterilised needle. Drops of spore suspension (3 μl) were then pipetted onto each of the berries to form a droplet either in the middle of the berry cheek for non-wounded berries or directly over the wound for wounded berries. The container was sealed and placed under fluorescent lighting on a 12 h light/dark cycle until required for sectioning.

Inoculation of Sauvignon blanc grapes was carried out in a similar manner for all developmental stages and included use of Botran fungicide and, for the berry samples, PVC mesh, as described above. Nine inflorescences or bunches were held with sterile forceps and sprayed with the spore suspension using a hand-held atomizer until runoff. At flowering and PBC, three intact inflorescences or bunches were placed in each of three separate containers. At veraison, three segments of approximately ten berries were cut from each bunch and all sections from a single bunch were placed in separate containers. Each container was sealed and placed under fluorescent lighting on a 12 h light/dark cycle until required for sectioning.

4.3.3. Fixing Sauvignon blanc samples

All Sauvignon blanc samples were fixed prior to sectioning for microscopy to satisfy GMO regulations for the transport of samples between facilities. Samples of three inflorescences or bunches were removed from their containers 24 h, 48 h and 5 d after inoculation: flowers or berries were removed and placed in a sterile container. A solution of 2.5 % formaldehyde in 0.1 M phosphate buffer was added to the container to completely cover the samples. The containers were placed in a vacuum chamber for 30 min to allow the formaldehyde to displace any air pockets within the sample. Containers were then stored at 4°C until required for sectioning.

4.3.4. Tissue sectioning and slide preparation

Crimson Seedless grapes were sectioned 48 h and 72 h after inoculation. Square blocks of berry tissue (5 mm^2 × 2 mm deep) were taken from around the inoculation site using a flame-sterilised scalpel. Where tissue was exhibiting rot symptoms, care was taken to sample tissue with no aerial hyphae. Tissue blocks were placed with the berry skin facing upwards in a custom-built microscope slide chamber (Figure 4.2). Transverse sections (5 mm × 2 mm × 2 mm deep) were taken from the area immediately adjacent to the inoculation site using a flame-sterilised scalpel and placed in the slide chamber with the berry skin facing the sides of the chamber.

Sauvignon blanc samples were removed from formaldehyde after 40 d, 19 d and 12 d for the flowering, PBC and veraison samples, respectively. Fixing duration was determined by vine phenology and availability of the confocal microscopes, as samples collected at a particular growth stage could not be visualised until the confocal microscopes were available for use. Samples were placed in a glass beaker containing 0.1 M phosphate buffer. After 20 min, samples were transferred to a fresh beaker containing 0.1 M phosphate buffer. This step was then repeated. For each inoculation time, 3–7 whole flowers were placed in a custom-built microscope slide chamber (Figure 4.2). For PBC and veraison bunches, berries were removed from the rachi and cut in half transversely using a sterile

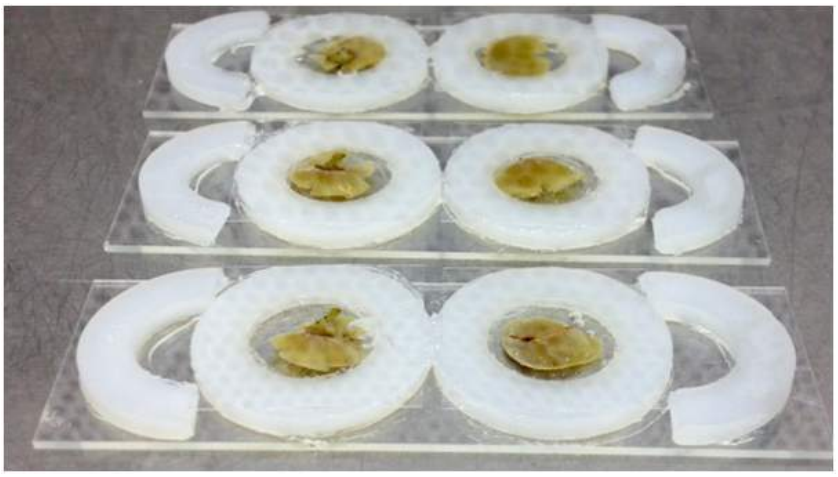

Figure 4.2. *Vitis vinifera* Sauvignon blanc berries collected at pre-bunch closure in slide chambers used for confocal microscopy. Chambers were constructed from nylon washers (24 mm external diameter, 12 mm internal diameter, 2 mm thick) bonded to a glass slide using co-polymer sealant (All Clear, Selleys, Auckland, New Zealand). Each chamber containing the berry or flower sample was filled with sterile water and a glass cover slip (22 mm^2) was sealed on top using co-polymer sealant thinned with mineral turpentine. Half washers were affixed near the ends of the slide to support the slide in the microscope.

scalpel. Four cuts were made to each berry half from either the torus or stylar end, depending on berry half, to the outer edge in order to allow the berry half to lie as flat as possible in the slide chamber (Figure 4.2). Excess pulp was removed from veraison berries to allow the berry halves to fit in the slide chambers. Each berry half was placed in a single slide chamber with the berry skin facing upwards.

4.3.5. Microscopy

Crimson Seedless samples were visualized immediately after tissue sectioning and slide preparation. Confocal microscopy was performed using an Olympus FluoView FV10i (Olympus Corporation, Tokyo, Japan) and diode lasers with an excitation wavelength of 473 nm and an emission wavelength of 510 nm for detection of GFP fluorescence, and excitation at 559 nm and emission at 600 nm for detection of grape skin cuticle auto-fluorescence. Differential interference contrast (DIC) images were collected using white light. Water immersion objectives were used with a working distance of 220 μm at maximum magnification.

Sauvignon blanc samples were visualised with stereo microscopy using a Leica FLZIII Stereo fluorescence microscope (Leica Microscopy Systems Ltd, Heerbrugg, Switzerland) prior to fixing. Sauvignon blanc samples needed to be transported to an external facility for confocal microscopy and were therefore fixed in formaldehyde to comply with regulations about the transport of a genetically modified organism. The fixation process for all Sauvignon blanc samples was longer than planned because of conflicts between the timing of vine phenology and the corresponding availability of the confocal microscopes, which were sometimes unavailable for weeks after sample collection. After samples had been fixed, sectioned and sealed in the slide chamber (Figure 4.2) they were transported on ice to the confocal microscope facility where they were visualized between 18 h and 48 h after sectioning. Flowers were visualised using a Leica TCS SP5 confocal microscope and berries were visualised on an Olympus Fluoview FV1000 confocal microscope mounted on an Olympus IX81 inverted microscope. Both confocal microscopes used an argon laser with an excitation wavelength of 488 nm and emission wavelengths of 501–551 nm for detection of GFP fluorescence and 651–751 nm for detection of grape skin cuticle auto-fluorescence.

Coverslips were removed from Sauvignon blanc PBC berry slides and a single drop of 1% lactophenol cotton blue was added to the slide chamber. The chambers were

sealed with a new coverslip and the tissues observed with the aid of a Nikon Eclipse E600 brightfield light microscope (Nikon Corporation, Tokyo, Japan).

All images produced by microscopy were processed using ImageJ (v1.48b; National Institutes of Health, Bethesda, MD, USA). Two-dimensional projection images of z-stacks were produced using the maximum projection method: the intensity of the brightest pixel was found for each equivalent pixel in a series of images prior to combining them into a single image (Sheppard and Shotton 1997).

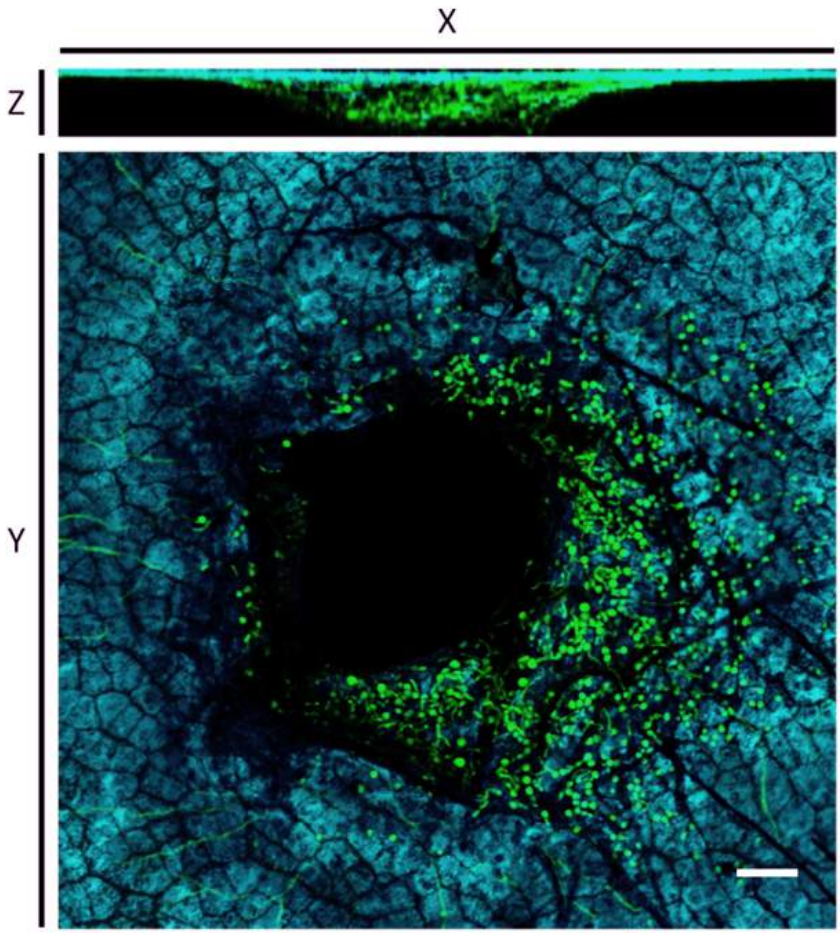

Figure 4.3. Orthogonal views of green-fluorescent protein (GFP)-expressing *Botrytis cinerea* (green) spores around a wound in a Crimson Seedless table grape berry 48 h after inoculation. Z-axis has been lengthened five-fold. Scale bar = 100 μm.

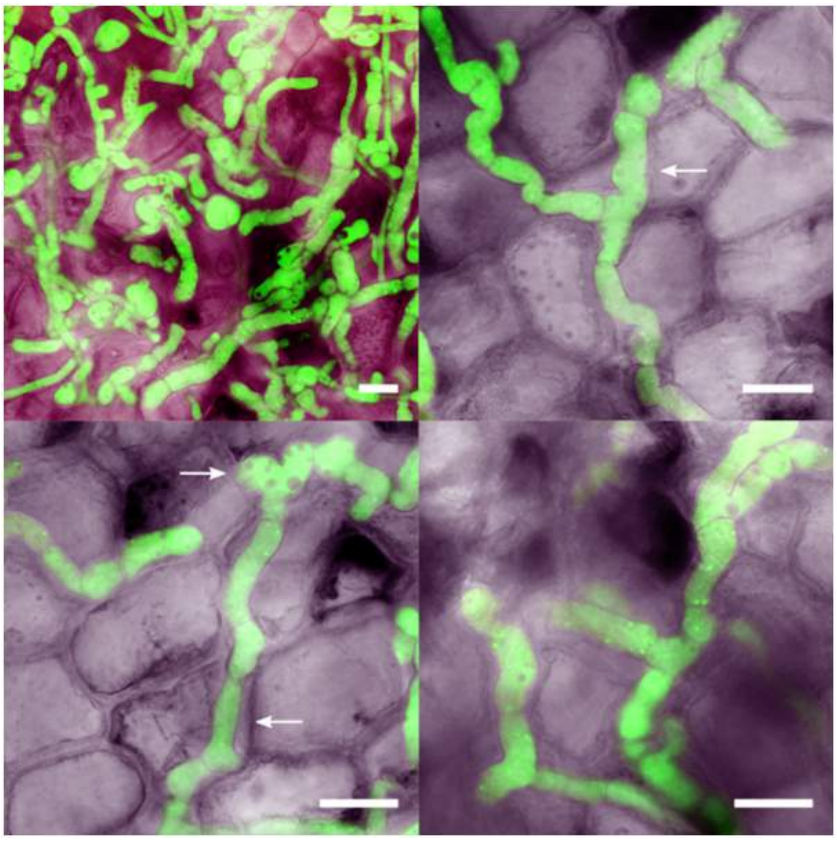

Figure 4.4. Green-fluorescent protein (GFP)-expressing *Botrytis cinerea* growing under the cuticle of Crimson Seedless table grape berry tissue 72 h after inoculation. Plant cells visualised using differential interference contrast (DIC). Arrows indicate possible intracellular growth. Scale bars = 20 μm.

4.4. Results

4.4.1. Crimson Seedless berries

Spores exhibiting GFP fluorescence were present on non-wounded Crimson Seedless grape berries, but no germination of these spores was observed. On wounded berries, fluorescing spores were only observed close to wounds and the majority of germinating spores were within approximately 200 μm of the wound's edge (Figure 4.3). The area covered by spores appeared to be smaller than the initial water droplet resulting from the inoculations; however, it is not known precisely how the spores settled within the water droplet. Mycelial growth within these wounded berries appeared to originate from within the wounds. No hyphae could be traced back to a germinating spore to confirm whether infection had occurred through the cuticle or directly into subcutaneous tissue from within the wound. The majority of hyphal growth observed within tissues

was intercellular with some possible intracellular growth (Figure 4.4) and was almost exclusively found between the cuticle and the epidermis, with some enlarged hyphae growing below the epidermis (Figure 4.5). Little to no hyphal growth was observed > 60 μm below the cuticle (Figure 4.6).

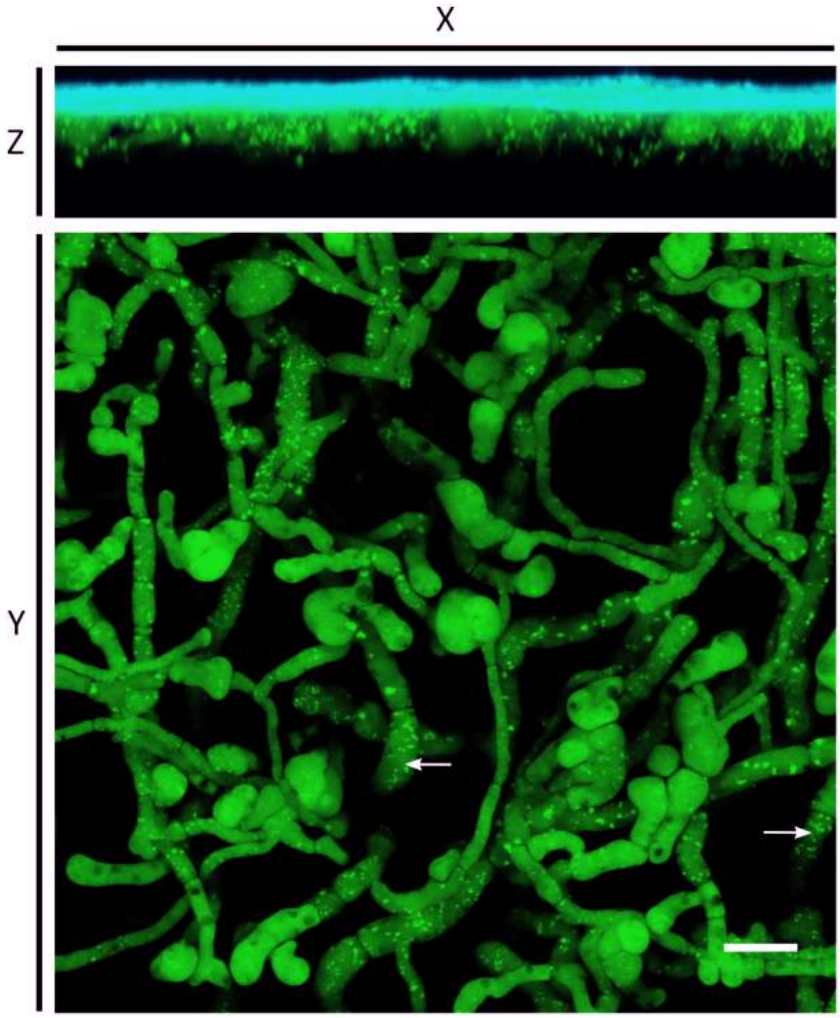

Figure 4.5. Orthogonal views of green-fluorescent protein (GFP)-expressing *Botrytis cinerea* (green) growing under the cuticle of Crimson Seedless table grape berry tissue visualized using confocal microscopy 72 h after inoculation. Z-axis has been lengthened five-fold to show mycelia growth in relation to the cuticle (cyan). Arrows indicate enlarged infection hyphae that appear to be growing down through the epidermis. Scale bar = 20 μm.

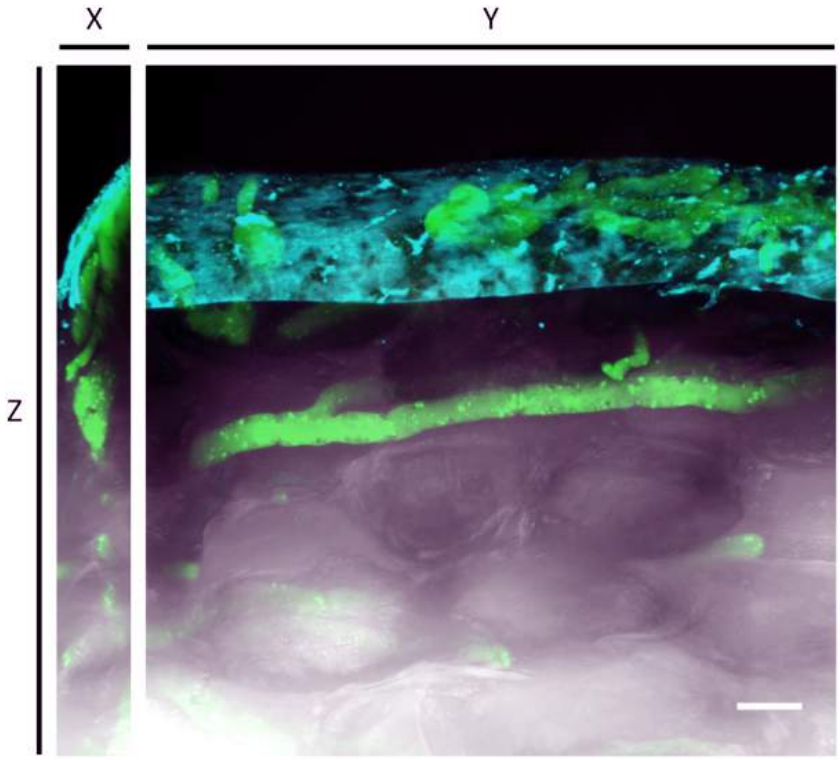

Figure 4.6. Orthogonal views of green-fluorescent protein (GFP)-expressing *Botrytis cinerea* (green) growing *in planta* in Crimson Seedless table grape berry tissue visualized using confocal microscopy 48 h after inoculation. The slanting cuticle (cyan) is caused by the sample being pressed against the coverslip. Plant cells visualised using differential interference contrast (DIC). X-axis has been lengthened two-fold. Scale bar = 20 μm.

4.4.2. Sauvignon blanc flowers

Stereo fluorescence microscopy revealed small colonies of GFP-expressing *B. cinerea* on floral parts of Sauvignon blanc grape flowers after 24 h (Figure 4.7a,b). Hyphal growth was observed on the majority of flowers after 48 h (Figure 4.7b,c). Fungal hyphae almost completely covered many flowers, including the styles and pedicels, after 5 d (Figure 4.7e,f). Auto-fluorescence of pollen was brighter than the fluorescence from GFP when the latter was observed (Figure 4.7c).

GFP fluorescence was weak in flowering samples at the time confocal microscopy was performed as samples were left too long in fixative. While increasing the gain on the microscope allowed hyphae to be visualised, this resulted in an increase in auto-fluorescence from plant cells. Adjusting the brightness and contrast in the image allowed hyphae to be distinguished from plant cells, however substantial image detail was lost in this process. Hyphal growth was found on only a few of the 24 h and 48 h flowering

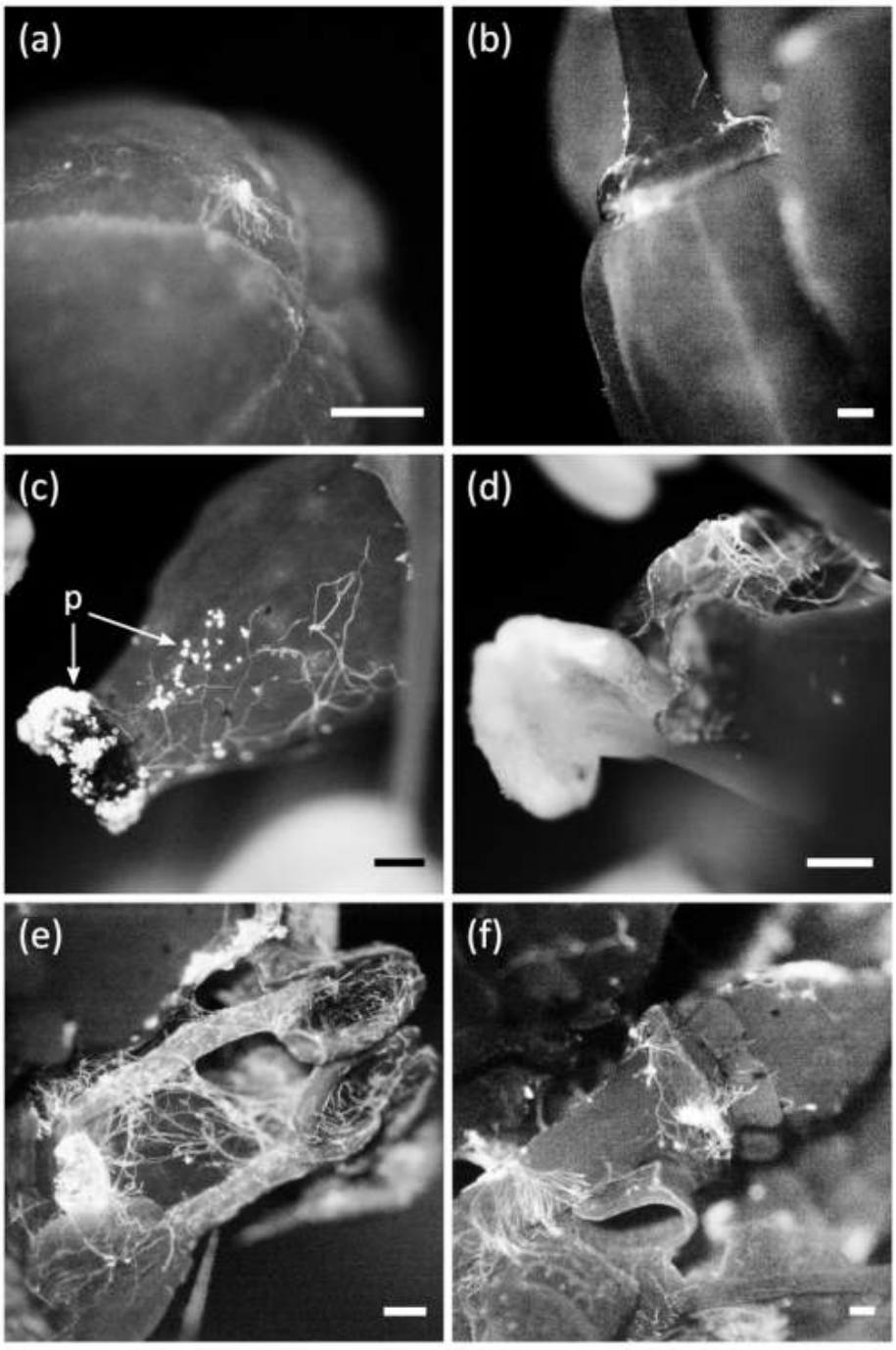

Figure 4.7. Sauvignon blanc inflorescences visualized with a stereo fluorescent microscope 24 h (a, b), 48 h (c, d) and 5 d (e, f) after inoculation with green fluorescent protein (GFP)-expressing *Botrytis cinerea*. Hyphae can be seen on the calex (a) and torus/pedicel (b, f) of pre-capfall flowers, the stamens (d, e) of post-capfall flowers and the developing berry (c, f). Autofluorescence of pollen (p) was brighter than the fluorescence of GFP-expressing fungal hyphae. Scale bars = 200 μm.

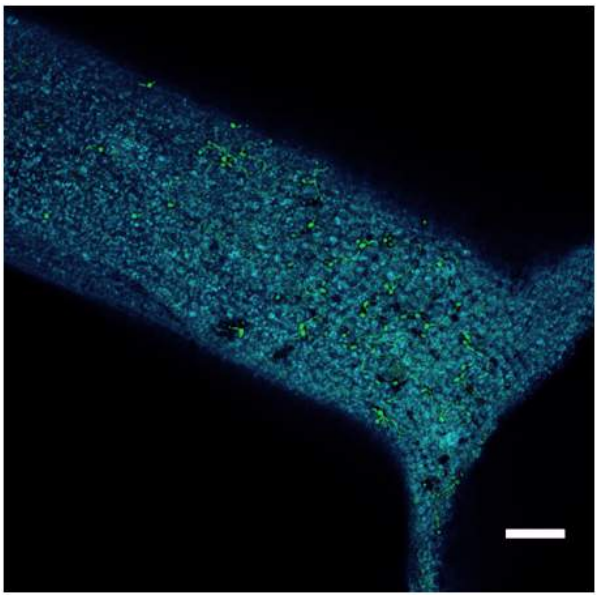

Figure 4.8 Germinating conidia of green fluorescent protein (GFP)-expressing *Botrytis cinerea* on pedicel of a Sauvignon blanc berry 24 h after inoculation. Scale bar = 100μm.

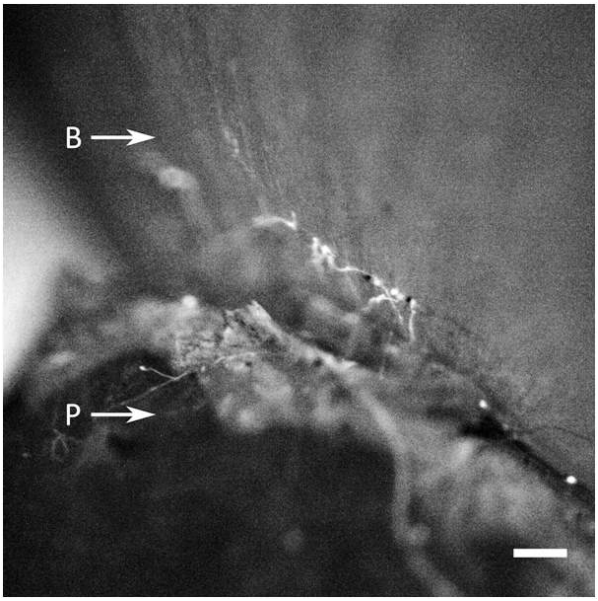

Figure 4.9 Pre-bunch closure (PBC) Sauvignon blanc grape berry (B) with pedicel (P) attached 5d after inoculation with green fluorescent protein (GFP)-expressing *Botrytis cinerea* exhibiting the most hyphal growth observed on all berries inoculated at this growth stage. Scale bar = 200μm.

samples and spore germination on these samples was observed predominantly on the torus and pedicel (Figure 4.8). Considerable hyphal growth was observed on 5 d flowering samples; however, the weak fluorescence meant it was not possible to determine whether this was surface or subcutaneous growth.

4.4.3. Sauvignon blanc berries at PBC

Very little hyphal growth was observed on PBC berries 24 h, 48 h or 5 d after inoculation, with hyphal growth covering an area of, at most, 0.15 mm^2 (Figure 4.9). No GFP

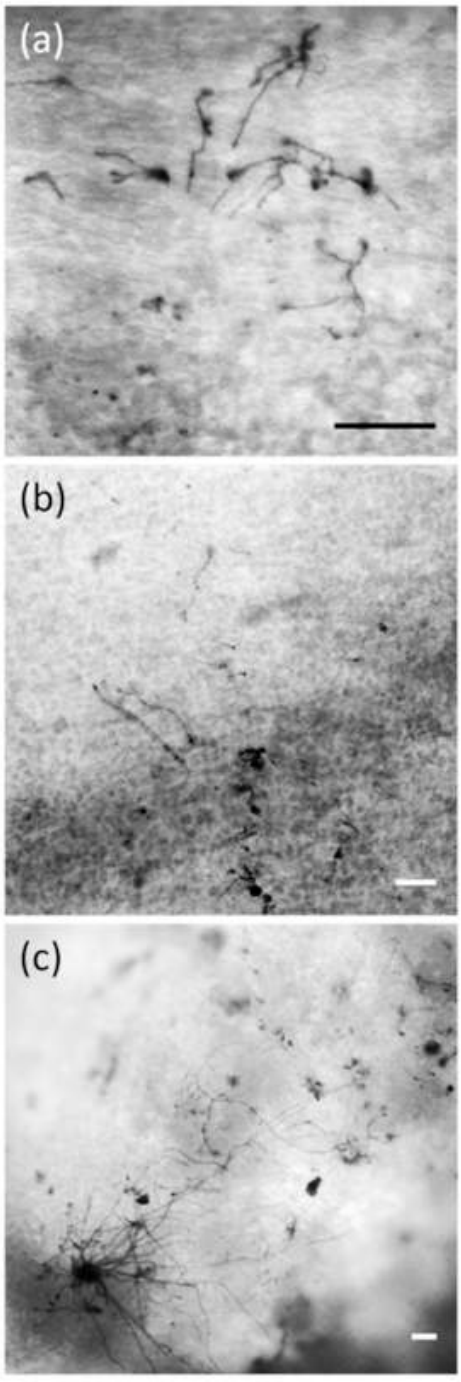

Figure 4.10 Skin sections of Sauvingon Blanc berries at pre-bunch closure (PBC) 24 h (a), 48 h (b) and 5 d (c) after inoculation with green fluorescent protein (GFP)-expressing *Botrytis cinerea*. Sections were stained with 1% lactophenol cotton blue. Scale bars = 100 μm.

fluorescence was observed by confocal microscopy in any of the PBC samples; it was unclear whether this was because of a lack of fungal growth or the samples being left in fixative too long. Lactophenol cotton blue revealed the presence of small amounts of fungal hyphae on the surface of the grape berries which may or may not have been *B. cinerea* (Figure 4.10).

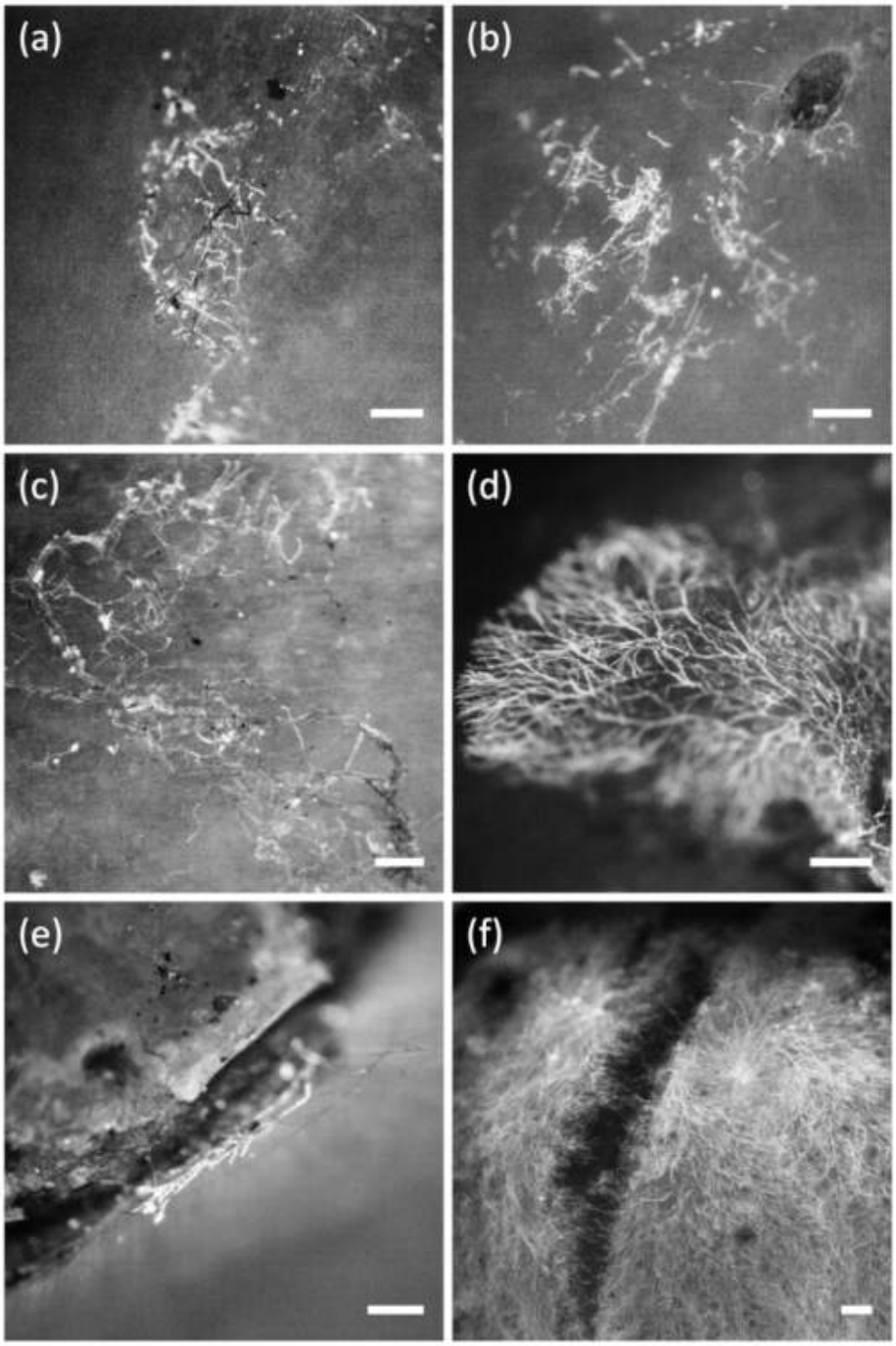

Figure 4.11. Sauvignon blanc berries at veraison visualized with a stereo fluorescent microscope 24 h (a, b), 48 h (c, d) and 5 d (e, f) after inoculation with green fluorescent protein (GFP)-expressing *Botrytis cinerea*. Mycelia can be seen growing on the unwounded berry cheek (a, b, c), wounded berry cheek (d, f) and receptacle area (e). Scale bars = 200 μm.

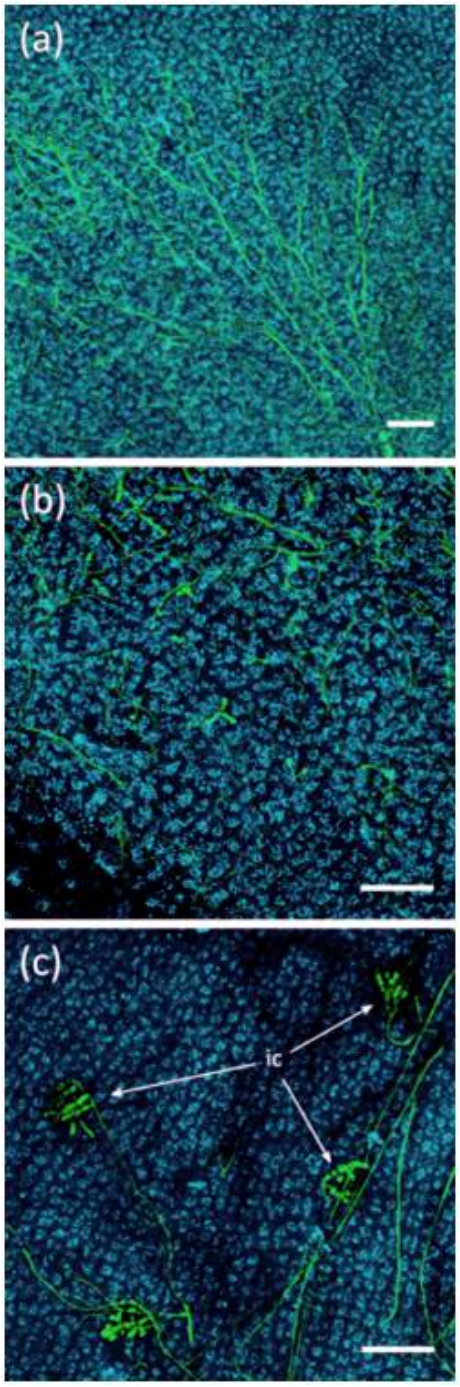

Figure 4.12 Wounded Sauvignon blanc berry at veraison, 5 d after inoculation with green fluorescent protein (GFP)-expressing *Botrytis cinerea* at veraison. Branching hyphae can be seen growing beneath the cuticle (a,b,c). Structures resembling infection cushions (ic) were only observed in one small area of one sample (c). Scale bars = 100 μm.

4.4.4. Sauvignon blanc berries at veraison

Hyphal growth was seen on all veraison berries at all times post-inoculation (Figure 4.11). The greatest area of berry skin surface covered by hyphal growth was observed on berries that had a wound present due to splitting (Figure 4.11d,f). Visually, growth on berries 5 d after inoculation and where splitting had not occurred was similar to

that observed 24 h and 48 h after inoculation (Figure 4.11a,c,e). Aerial hyphae on split berries radiated out from the wounds; in the area adjacent to this, hyphae appeared to be growing subcutaneously (Figure 4.12). Hyphal structures that appeared similar to infection cushions (Backhouse and Willetts 1987), based on morphology, were observed growing beneath the cuticle and the areas of cuticle directly above these structures was degraded (Figure 4.13).

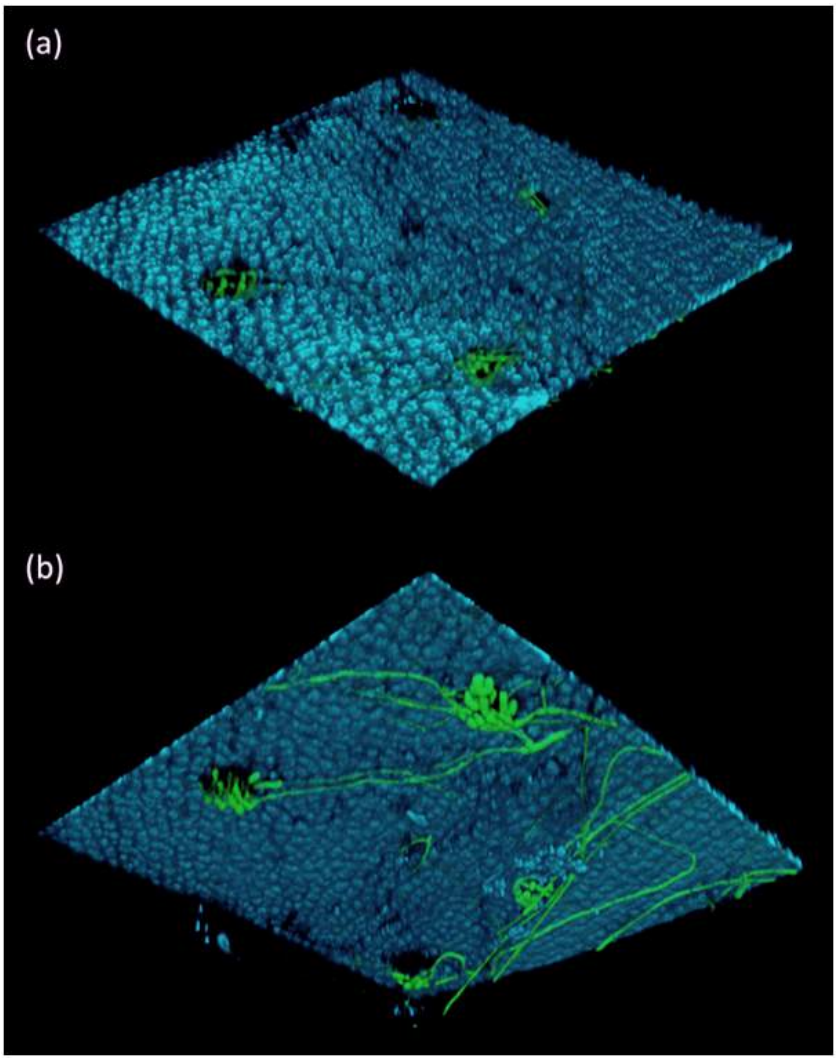

Figure 4.13. Three-dimensional reconstruction of Figure 4.12c showing green fluorescent protein (GFP)-expressing *Botrytis cinerea* mycelia (green) growing directly under the cuticle (cyan) of a wounded Sauvignon blanc grape berry at veraison 5 d after inoculation. (a) Berry cuticle surface. (b) Underneath cuticle.

4.5. Discussion

A number of critical aspects of the infection and growth of *B. cinerea* in grape flowers and berries have been observed through the use of a GFP-expressing strain of *B. cinerea* and a range of microscopic techniques, including confocal microscopy.

It was found that spores did not germinate on the mature Crimson Seedless table grape berries in the absence of wounding. The pattern of spore distribution around the wound suggested that most of the spore-containing droplet had been absorbed directly into the pulp through the wound, thereby bypassing the physical barrier provided by the berry skin. These observations confirm that wounding, which has previously been shown to increase incidence of infection in detached grape berries (Mundy et al. 2009), provided both a means for *B. cinerea* to enter the plant and an environment suitable for fungal growth inside the wound. On wounded berries, the frequency of germinated spores increased closer to the woundâĂŹs edge. Percentage germination of *B. cinerea* spores has been shown to increase in the presence of nutrients exuded by grape berries (Kosuge and Hewitt 1964) as well as plums and nectarines (Fourie and Holz 1998). The pattern of spore germination surrounding the wound is consistent with leakage of nutrients from the wound and the stimulation of germination of the spores closest to the wound.

The pattern of intercellular hyphal growth beneath the cuticle with enlarged hyphae penetrating deeper into the tissue of infected Crimson Seedless berries is consistent with what has previously been described in *Arabadopsis* (Govrin and Levine 2000) and onion (Clark and Lorbeer 1976). This pattern of primary localisation of hyphal between the cuticle and the epidermis with limited penetration deeper into the berry was observed in both transverse and tangential sections. Hyphal growth localised in this region could degrade the epidermis while leaving the pulp relatively intact. It is likely that this could cause the skin to separate from the pulp, which would be consistent with the slip skin symptoms that are sometimes observed (Nelson 1956; Hewitt 1974). Although intracellular growth may have occurred, it could not be confirmed in this study. Hyphae growing over or under a cell may appear to be intracellular in a maximum projection, as the image-processing technique flattened the z-stack into a 2-dimensional image. More precise host cell wall staining to visualise associated cell wall reactions, such as callose formation, would be required to determine the extent of intracellular growth (Harper et al. 1996).

Under the experimental conditions used here, the surface of some Sauvignon blanc flowers were colonised almost completely within a few days of inoculation. Germinating

spores were found on the surface of many floral parts, including the pedicel. Because of weak GFP fluorescence under confocal microscopy, the differentiation of hyphae from plant cells was not strong and it was not possible to determine if penetration of the epidermis had occurred. Therefore, it could not be determined if infections that could remain as latent infections were occurring in the flowers.

In contrast to the abundance of the pathogen on the surface of inoculated flowers, very little fungal growth was found on the surface of berries sampled at PBC using stereo fluorescence microscopy and none observed using confocal microscopy. Only a small amount of fungal growth was revealed through staining and brightfield microscopy. This lack of fungal growth may have been a feature of the stage of maturity of the berries, as reduced germination and infection rates have been reported previously in immature grape berries (Hill et al. 1981). The few germinating spores that were observed by light microscopy tended to be located towards the torus end of the berry cheek. This distribution of spores suggested that the spore suspension may have gravitated to the base of the berry following inoculation and is consistent with the observations of Viret et al. (2004) that showed a predominance of spores at the base of the berry. However, because of the low numbers of germinating spores observed in this study it was not possible to confirm the location of infection sites at this phenological stage.

Stereo fluorescence microscopy of berries inoculated at the veraison stage revealed considerable hyphal growth on the surface of all berries in which splitting had occurred following inoculation. The splitting was considered to be the result of swelling of the berries under the high humidity conditions in containers. Some growth was observed on non-wounded berries, but this was less common and was similar at 24 h, 48 h and 5 d after inoculation. This pattern of infection provided further evidence of the importance of wounding for the infection of mature berries. The intensity of GFP fluorescence was low in berries inoculated at veraison and hyphae were visualised by confocal microscopy in only a few samples, despite being seen on all wounded berries prior to being placed in fixative. Where GFP-expressing hyphae were observed, growth was intercellular and subcuticular similar to that observed in Crimson Seedless berries. An additional feature observed in one of the Sauvignon blanc berries was the presence of lobed structures resembling infection cushions. These appeared to be growing below the cuticle, an atypical position for infection cushions, which are usually a means of penetrating the skin from the surface. Where these structures were present, the cuticle appeared to have been degraded suggesting that their role may have been to facilitate the exit of the

fungus from the berry in order to produce aerial hyphae and subsequently conidiophores, although no such aerial hyphae were observed at the location of these structures.

Because of New ZealandâĂŹs regulatory constraints on the movement and handling of the GFP-modified strain of *B. cinerea* and the location of some of the confocal microscopes used in the study, it was sometimes necessary to kill the infected tissue by fixing in formaldehyde before transporting the material between sites. Where freshly harvested material was used, such as the Crimson Seedless samples, good definition of fungal hyphae on and within tissues was obtained. However, in circumstances when it was necessary to hold the fixed material for some time, it was found that the degree of fluorescence declined to the extent that, in some cases, it was barely possible to discriminate between the GFP fluorescence of the pathogen and the background fluorescence of the plant cells. Very little information is available on the effects of different fixatives or the duration in fixative on GFP fluorescence. This study showed that for visualising infection processes of GFP-modified fungi in plants by fluorescence microscopy, the best results will be obtained on fresh material or material that has only been held in formaldehyde for a short time.

This study demonstrated some aspects of the *in planta* growth of *B. cinerea* in grape flowers and berries not previously described. The inoculation of detached inflorescences and berries may not necessarily reflect infections in the field. For observation of the location and development of latent infections, inoculation of flowers and berries on whole plants, followed by tissue sampling and microscopy, preferably without fixation, is required. Under New Zealand‘s GMO regulations, this could be achieved by inoculating vines or flowering cuttings with GFP-expressing *B. cinerea* in a suitable containment glasshouse to allow sampling of the developing berries as infections progressed naturally. Samples should be examined periodically, allowing latent infections to be localised and aspects of *in planta* growth such as hyphal growth rates and survival of latent infections to be investigated.

4.6. Acknowledgements

This work was supported by the The New Zealand Institute for Plant & Food Research and the Tasmanian Institute of Agriculture (TIA), University of Tasmania. We thank Matt Templeton (Plant & Food Research) and Gregor Kolbe (University of Auckland) for supplying the GFP-expressing *B. cinerea* strain, and Ian Hallett (Plant & Food

Research), Manfred Ingerfeld (University of Canterbury) and Barry O'Brien (University of Waikato) for their assistance with sample preparation and microscope operation.

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Chapter 5

Near- and mid-infrared spectroscopy for the quantification of botrytis bunch rot in white wine grapes

Published in:

http://www.impublications.com/content/journal-near-infrared-spectroscopy

Gareth Hill, Katherine Evans, Robert Beresford, and Robert Dambergs (2013). Near and mid-infrared spectroscopy for the quantification of botrytis bunch rot in white wine grapes. *Journal of Near Infrared Spectroscopy* Vol. 21 (6) 467-475.

DOI: 10.1255/jnirs.1083

6

Comparison of methods for the quantification of botrytis bunch rot in white wine grapes

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Originally formatted for *Plant Disease*.
Accepted by *Australian Journal of Grape and Wine Research* on May 14th, 2014.

6.1. Abstract

Quantification of botrytis bunch rot (BBR) in wine grapes, caused by *Botrytis cinerea*, is commonly done by visual estimation of the percentage area of individual bunches displaying BBR. The accuracy of visual estimation was compared with those of four other quantification methods: digital image analysis, near-infrared (NIR) and mid-infrared (mid-IR) spectroscopy, and quantitative PCR (qPCR). Visual estimation was found to vary significantly ($P < 0.05$) among assessors, suggesting that assessor training or correction is required to ensure adequate accuracy. Image analysis software (RotBot) was developed to measure BBR severity from digital images of grape bunches using pixel hue. All quantification methods showed significant relationships ($P < 0.05$) with visual estimation. NIR and mid-IR spectroscopy were poorly related to visual estimates at lower BBR severities (e.g. $< 25\%$) and would require further calibration to be suitable alternatives. Quantitative PCR was the most accurate method but it is too labour intensive to be considered useful for routine use in the vineyard. RotBot was found to be the most suitable and practical alternative to visual estimation, as it is an objective measure and requires no specialised equipment. The issue of identifying a suitable reference method for alternative quantitative measures of BBR severity is discussed.

6.2. Introduction

Botrytis bunch rot (BBR), caused by *Botrytis cinerea* Pers., can decrease grape yield and wine quality (21). Depending on the end use of the grapes, wineries often impose price penalties if the mean severity exceeds a certain percentage, often 3–5%, because of the impact *B. cinerea* can have on the winemaking process (15). Control costs and lost income from severe epidemics make BBR one of the most economically important diseases in wine grapes (27). Quantification of BBR on wine grapes is commonly done by visually estimating the percentage bunch area with symptoms in a sample of bunches and then calculating the mean severity of the sample. Visual estimation has been shown to be inaccurate for diseases in other crops because of systematic bias in measurements made by assessors (1, 3, 34). A severity assessment key and the electronic tool Bunch Rot Assessment Trainer (BRAT) have been developed to improve accuracy and reduce variability between assessors (17). While such tools have been shown to improve the accuracy of assessments (22), there is a need for an objective quantification method that is cost effective and practical.

Spectroscopic techniques, such as near-infrared (NIR) and mid-infrared (mid-IR) spectroscopy, have the potential to provide objective, rapid and cost-effective methods for the quantification of BBR. Both NIR and mid-IR spectroscopy are currently used in the wine industry to measure alcohol and anthocyanin content in wine (8, 9). These techniques are being explored for other quality parameters because of the ability of one scan to be analysed by chemometrics for multiple chemical and biophysical properties (14). This approach also shows potential not only for wine, but also for grape must or whole grapes, where it has been used successfully to measure total soluble solids (°Brix), reducing-sugar content and pH (13, 16). Both NIR and mid-IR spectroscopy have been shown to be successful methods for quantifying *B. cinerea* in homogenised wine grapes; however, the accuracy of both methods for the severity ranges most commonly found in commercial vineyards was too low for the methods to be considered useful without further calibration (18).

Image analysis of digital photographs can also be used for quantifying plant diseases (4). Digital images are commonly stored as RGB (red, green, blue) images, meaning that each pixel is a mix of the primary colours red, green and blue, with the amount of each colour in a pixel having a value of 0–255 (4). Pixels can also be defined by their HSV (hue, saturation, brightness) characteristics. Hue is the colour of the pixel with a range of 0–360, saturation is the amount of colour with a range of 0–100, and value is the brightness of colour, from pure hue to black, with a range of 0–100; these components can be easily calculated from RGB values (23). Plant disease severity can be quantified using image analysis by assigning ranges for pixels depicting healthy or diseased tissue using either the RGB or HSV colour systems or one of the components within those systems (4). The hue of pixels in an image is not affected by differences in light conditions, arising from shading, time of day, weather, the reflectivity of plant surfaces and the exposure of the image. These features might explain why hue has been found to be the most effective characteristic for distinguishing pixels belonging to diseased and healthy plant tissue (2, 6, 10, 28).

Previously, there has been a focus on developing molecular methods for the detection of plant pathogens using the polymerase chain reaction (PCR; 20). Rigotti et al. (24) designed primers specific to *B. cinerea*, although they later redesigned the primers to allow better specificity (25). While PCR allows specific detection of *B. cinerea*, this does not solve the problem of quantifying the amount of fungus present. Real-time quantitative PCR (qPCR) allows the amount of DNA in the reaction to be quantified. With this information, the amount of fungal mass in the original sample can be estimated through

Table 6.1. *Vitis vinifera* samples used to compare four quantification methods for botrytis bunch rot in wine grapes. A dash indicates where a quantification method was not applied to a sample.

Samples				Quantification method			
Collection Date	Vineyard location	Variety	Sample type	Visual estimation	RotBot	Infrared spectroscopy	qPCR
22 March 2012	Tamar Valley, Tasmania, Australia	Riesling	30 bunches used to manufacture six severity categories	-	-	-	Calibration
21 May 2012	Auckland, New Zealand	Sauvignon blanc	26 bunches digitally photographed	Assessor comparison	Software development and comparison with assessors.	-	-
21 May 2012	Auckland, New Zealand	Sauvignon blanc	10 symptomless leaves	-	-	-	Extracted DNA used as positive control
3 April 2013	Tamar Valley, Tasmania, Australia	Riesling	66 bunches divided into six severity groups	Comparison with other quantification methods	Evaluation of bunch side(s) required Compared with visual estimation	Compared with visual estimation	Compared with visual estimation

application of standardisation protocols (12, 32). Using primers designed by Rigotti et al. (24), a qPCR protocol has been developed for quantification of *B. cinerea* in grape berries using DNA from artificially inoculated, immature berries (26). This method, together with an earlier assessment of qPCR (5), indicate that further development of this technique is needed for application in the vineyard.

The aim of this study was to use grape bunches infected naturally with *B. cinerea* to investigate the accuracy of visual estimation and to compare it with each of the alternative methods, NIR and mid-IR spectroscopy, digital image analysis and qPCR, to determine if any of these methods could be useful alternatives. This study also presents further modifications to DNA extraction and duplex qPCR techniques reported previously (26).

6.3. Materials and Methods

6.3.1. Sample collection

Samples of grape bunches with and without BBR symptoms were collected from vineyards in Tasmania and New Zealand in 2012 and 2013 (Table 6.1).

On 22 March 2012, 30 bunches of *Vitis vinifera* L. Riesling (RS) at the modified Eichhorn-Lorenz (E-L) growth stage 37 (pre-harvest; 7) were collected from a vineyard in the Tamar Valley, Tasmania (-41.1829, 146.8841). For each grape bunch, disease severity was estimated visually as the percentage of berries or tissue exhibiting BBR symptoms when viewed from one side of the bunch with the aid of a standard area diagram (17). After scoring, each bunch was placed into individual re-sealable plastic bags prior to same-day road transport to the laboratory and storage at -20°C until required. After thawing, each berry from each bunch was detached from the pedicel and placed in one of two plastic trays: 1) berries with no visible BBR symptoms ('healthy') and 2) berries with BBR symptoms ('rotted'). Healthy and rotted berries were combined by weight into containers to achieve manufactured BBR severities of 0, 5, 10, 25, 50 and 100%. Three containers with a total of 75 g of berries (approximately 100 berries) were collected per designated BBR severity percentage, giving a total of 18 containers of manufactured samples. The containers were sealed and stored at -20°C.

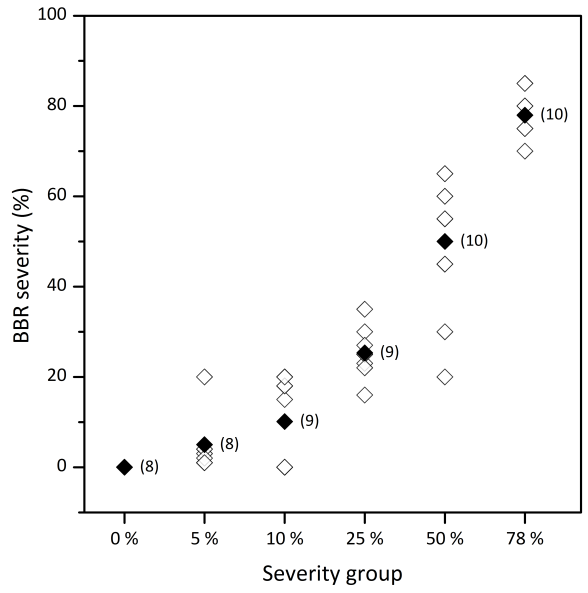

Figure 6.1 Botrytis bunch rot (BBR) severities for each grape bunch (hollow diamond) used to generate the 2013 severity groups compared with the mean for each group (solid diamond). Number of bunches in each group shown in parentheses next to group means.

For grape DNA extraction, 10 young *V. vinifera* Sauvignon blanc leaves with no visible symptoms of *B. cinerea* infection were collected at E-L 41 (post-harvest) on 21 May 2012 from a research vineyard in Auckland, New Zealand (-37.2038, 174.8636) and then stored in polythene bags at 4°C overnight before DNA extraction. For image analysis, 26 bunches with various percentages of BBR severity were collected from the same vineyard on the same date. These bunches were placed individually on a piece of blue cardboard and photographed with a digital camera (Lumix DMC-G2, 4 megapixels, automatic setting; Panasonic Corporation, Osaka, Japan). A single *B. cinerea* strain was isolated from one of the infected bunches and grown on malt extract agar (MEA) for 10 d under fluorescent lights programmed for a 12 h light/dark cycle.

On 3 April 2013, 66 Riesling bunches were collected at the same E-L growth stage from the same Tamar Valley vineyard as the previous season. Each bunch was scored for BBR severity as above, placed in a blue plastic tray and photographed with the same digital camera and settings as above. The bunch was turned over to photograph the other side. Each bunch was placed into an individual re-sealable plastic bag prior to same-day road transport to the laboratory and storage at -20°C until required. After thawing, bunches were grouped according to BBR severity to give the following mean BBR severities: 0, 5, 10, 25, 50 and 78% (Figure 6.1). For each group, the berries were detached from the pedicels, mixed and divided into six subsamples of approximately 200 berries and placed in 200-ml containers. The containers were sealed and stored at -20°C.

6.3.2. Assessor comparisons

Twenty of the digital images of Sauvignon blanc bunches with a range of BBR severities were scored by 20 assessors with moderate to extensive experience in visual assessment of BBR severity, including researchers, vineyard managers and vineyard consultants. For each image, assessors were asked to estimate visually the percentage of the bunch with BBR symptoms. Six months later, the same twenty images were randomised and 12 of the original 20 assessors were asked again to estimate the percentage of each bunch with BBR symptoms.

6.3.3. Digital image analysis

The digital images of diseased and healthy bunches were analysed with code written using Microsoft Visual C# Studio Express Edition (version 10.0 RTMRel; Microsoft Corporation, Redmond, WA, USA). This code retrieved the hue value of each pixel and calculated the frequency of pixels in each image with each possible hue. Hue distributions were calculated for each of the 26 digital images of Sauvignon blanc bunches collected on 21 March 2012. Images were cropped to the edges of the bunch so as to include only the grape bunch, with no background. To investigate the differences in hue distributions for healthy and diseased bunches, images were then scored visually for BBR severity (%) and separated into two groups: bunches with $\leq$ 25% BBR severity, and bunches with $>$ 25% BBR severity. The mean hue distributions of the two groups and for all bunches were then compared. Hue distribution information obtained from the Sauvignon blanc bunch images was used to determine hue ranges attributable to diseased and healthy tissue. This information was used to write software ('RotBot') using Microsoft Visual C# Studio Express Edition for measuring BBR severity in digital images of grape bunches by calculating the number of pixels attributable to diseased tissue as a percentage of total pixels attributable to diseased tissue and healthy tissue.

RotBot was used to measure BBR severity in the same 20 digital images of Sauvignon blanc bunches sent to the assessors. A linear regression was performed using the BBR severity measured by RotBot versus the mean BBR severity estimated visually by each assessor for each image.

Digital images of Riesling bunches collected in 2013 were used to test whether it was necessary to capture an image of both sides of a grape bunch to avoid bias when measuring BBR severity. RotBot was used to calculate mean BBR severity using images of one side of the bunch, images of the other side of the bunch, or the mean from images of both sides. To investigate any potential effect of the order in which the two sides were photographed, the two images captured for each bunch were designated as the first and second sides using the order in which the images were captured and also by randomly designating the two sides. Means were calculated using both the original order and the random order.

BBR severity of the six severity groups from 2013 grape bunch samples was measured by RotBot using images of both sides of each of the bunches that made up each group.

6.3.4. DNA extraction

Total nucleic acids were extracted from all containers of Riesling berries collected in 2012 and three containers of Riesling berries from each BBR severity category collected in 2013. DNA was also extracted from leaves of Sauvignon blanc and *B. cinerea* mycelium.

Each container of berries was removed from the freezer and the berries transferred to an unbleached calico bag which was then immersed in liquid N_2 for 20 s. The bag of berries was then transferred to a bench and the berries crushed with a rubber mallet for 30 s. The berry fragments were emptied from the bag into a mortar and pestle cooled with liquid N_2 and ground to a fine powder. Approximately 100 mg of ground tissue was then added to each of three 1.5-ml microfuge tubes cooled previously in liquid N_2. The tubes containing the ground tissue were stored at -20°C.

Leaf tissue and *B. cinerea* mycelium were also ground to a fine powder using a mortar and pestle and liquid N_2. *Botrytis cinerea* mycelium was scraped from the surface of an MEA plate using a glass cover slip. Approximately 100 mg of ground tissue (leaves or mycelia) was transferred to each of three pre-cooled 1.5-ml microfuge tubes. The tubes were stored at -20°C.

The procedure for DNA extraction was modified from that of Saito et al. (26). Tubes containing ground tissue were removed from the freezer and placed into liquid N_2. Each tube was removed from the liquid N_2 and 500 μl of extraction buffer, warmed to 65°C, was added and the tube was inverted until the tissue was mixed with the buffer. The extraction buffer contained 5 M NaCl, 1 M Tris-HCl (pH 8.0), 0.5 M EDTA (pH 8.0), 2% w/v CTAB and 1% w/v PVP40. Tubes were then placed in a 65°C water bath and inverted every 5 min. After 20 min, 500 μl of chloroform:isoamyl alcohol (24:1) was added to each tube and the tubes placed on a rotating mixer for 10 min at room temperature, followed by centrifugation for 10 min at 20,160 $\times$ *g*. The supernatant from each tube was transferred to a fresh 1.5-ml microfuge tube, 500 μl of chloroform:isoamyl alcohol (24:1) was added and the tubes rotated and centrifuged as before. The supernatant was transferred to a new tube followed by the addition of 30 μl of 10% (w/v) CTAB and 500 μl of chloroform:isoamyl alcohol (24:1). The tubes were placed back on the rotating mixer for 10 min. Centrifugation and supernatant transfer was repeated as above, followed by the addition of 150 μl of 5 M NaCl and 1 ml of ice-cold 95% (v/v) ethanol. The tubes were held on ice for 1 h then centrifuged for 15 min at 20,160 $\times$ *g*. The ethanol was poured off carefully and the DNA pellet suspended in 1 ml of ice-cold 70% (v/v) ethanol. The tubes were placed on a rotating mixer in a 4°C cool room for 20 min. The

tubes were then centrifuged for 10 min at 20,160 × g and the ethanol poured off and evaporated by placing the DNA pellets in a DNA Mini vacuum concentrator (Thermo Fisher Scientific Inc., Wilmington, DE, USA) at 45°C for 3 min.

The DNA pellet was resuspended in 50 μl sterile distilled H_2O (dH_2O) and the concentration (ng/μl) of DNA measured using a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, DE, USA). All samples were diluted in sterile dH_2O to 10 ng/μl and stored in the freezer at -20°C.

6.3.5. Quantitative PCR

Two previously published primer sets were used in qPCR reactions. *Botrytis cinerea*-specific primers (Bc3_F: 5'-GCTGTAATTTCAATGTGCAGAATCC-3'; Bc3_R: 5'- G GAGCAACAATTAATCGCATTTC-3') were targeted to an intergenic spacer region between the 28S and 18S genes (GenBank: AM233400.1) and produced a 95 base pair (bp) amplicon (30). *Vitis vinifera*-specific primers (Res_F: 5'-CGAGGAATTTAGAA ACGCTCAAC-3'; Res_R: GCTGTGCCAATGGCTAGGA-3') were targeted to the resveratrol synthase I gene (GenBank: JN858960.1) and produced a 63 bp amplicon (31).

Two previously published probes were modified for use in duplex qPCR reactions where *B. cinerea* DNA and *V. vinifera* DNA were amplified simultaneously. The *B. cinerea*-specific probe (Bc3_P: 5'-FAM-AGGTCACCTTGCAATGAGTGG-BHQ-1-3') was targeted to the amplicon produced by the Bc3 primer set and was modified from the method of Suarez et al. (30) by adding three bp to the 5' end in order to raise the melting temperature. The *V. vinifera*-specific probe (Res_P: 5'-CAL FLUOR Red 610-T GCCAAGGGTCCGGCCACC-BHQ-2-3') was targeted to the amplicon produced by the Res primer set and was modified from the method of Valsesia et al. (31) by changing the 5' label to CAL FLUOR Red 610 to contrast better with the FAM reporter used on the Bc3_P probe.

All qPCRs were performed in an Eco real-time PCR machine (Illumina Inc., San Diego, CA, USA). Reaction volumes were 10 μl and reactions were carried out in 48-well plates. Reaction conditions for all qPCR reactions were 3 min at 95°C followed by 40 cycles of 95°C for 15 s and 60°C for 45 s.

Two qPCRs were carried out to produce standard curves and verify the method: one with *V. vinifera* DNA from leaves as the template, and one with *B. cinerea* DNA from

mycelium as the template. For each assay, a ten-fold dilution series of template DNA was used: 10, 1, 0.1, 0.01 and 0.001 ng. Each dilution was replicated three times. A dH_2O control was included in triplicate in each assay. The reaction mix for monoplex qPCR was as follows: 1 x PerfeCTa® SYBR® Green FastMix® (Quanta BioSciences, Inc., Gaithersburg, MD, USA), 0.5 μM of each primer (Bc3_F and Bc3_R or Res_F and Res_R) and 2 μl of template DNA (various concentrations).

Duplex qPCR assays were carried out with *B. cinerea* DNA mixed with either *V. vinifera* DNA or dH_2O to investigate any potential inhibition of *B. cinerea* DNA amplification by *V. vinifera* DNA. Duplex qPCR assays were also carried out with total DNA extracted from the 2013 manufactured samples. The reaction mix for duplex qPCR was as follows: 1 x PerfeCTa® qPCR ToughMix®(Quanta BioSciences, Inc., Gaithersburg, MD, USA), 0.5 μM of each Bc3 primer, 0.1 μM of each Res primer, 0.15 μM Bc3_P probe, 0.15 μM Res_P probe, 20 ng template DNA. All duplex qPCR plates contained a reference DNA sample in triplicate consisting of 1 ng *B. cinerea* DNA and 10 ng *V. vinifera* DNA, and a dH_2O control in triplicate.

6.3.6. Homogenisation

Three subsamples of berries from each of the six BBR severity categories for the 2013 samples of Riesling berries were homogenised for measurement of total soluble solids content (°Brix) and for analysis by infrared spectroscopy. The containers were removed from the freezer and stored at 4°C overnight to allow the berries to thaw before homogenisation. Samples were homogenised using a Grindomix GM 200 (Retsch, Haan, Germany) set at 8,000 rpm for 20 s. A 4-ml volume of homogenate was pipetted into a 5-ml glass vial with a rubber stopper and 1 ml was pipetted into each of two 1.5-ml microfuge tubes. These samples were stored at -20°C. The grinding jar was washed with water and dried with paper towels between each sample.

6.3.7. Soluble solids

One set of microfuge tubes containing 1 ml of grape homogenate was removed from the freezer and placed on the laboratory bench to thaw at room temperature. Once thawed, each entire sample was centrifuged at 7,000 $\times$ g for 1 min. The supernatant was then

pipetted onto the glass reading surface of a Pal-1 digital refractometer (Atago Co., Ltd; Tokyo, Japan) and the soluble solids concentration (°Brix) was recorded.

6.3.8. Infrared spectroscopy

All glass vials containing homogenised berry samples were removed from the freezer and stored at 4°C overnight to allow the homogenates to thaw before analysis by NIR spectroscopy. Glass vials were placed in the carousel of a Bruker MPA FT-NIR machine (Bruker Optics GmbH, Ettlingen, Germany) for automatic processing. Samples were scanned using reflectance NIR, and visual BBR severity predicted according to the method of Hill et al. (18).

One set of microfuge tubes containing 1 ml of grape homogenate was removed from the freezer and placed on the laboratory bench to thaw at room temperature. Once thawed, each entire sample was pipetted onto the crystal reading surface of a Bruker MPA FT-MIR machine (Bruker Optics GmbH, Ettlingen, Germany). Samples were scanned using reflectance mid-IR and visual BBR severity predicted according to the method of Hill et al. (18).

6.3.9. Statistical analyses

All statistical analyses were carried out using GenStat 14^{th} Edition software (VSN International, Hemel Hempsted, UK).

Analysis of variance (ANOVA) was used in comparisons of mean BBR severity from each assessor and also in comparisons of mean BBR severity calculated using RotBot with images of one or both sides of each bunch, in capture order and random order, and visual estimation. Bunch number was used as a blocking effect in both analyses to account for high variability in BBR severity among individual bunches.

Pathogen coefficient (PC) was calculated for all duplex qPCR runs as the ratio of quantification cycle (Cq) for *B. cinerea* DNA to that for *V. vinifera* DNA as described by Saito et al. (26). Nine PC values were obtained from each severity category/group: three containers of berries, three subsamples of ground tissue from each container, and three replicates of DNA from each subsample of ground tissue. Sources of variation were investigated through covariance analysis by fitting a mixed model to PC with BBR

severity as a fixed effect and all levels of replication (berries, ground tissue and DNA) as random nested effects.

The ratio of standard error of prediction to standard deviation of the reference (*RPD*) was calculated for predictions of BBR severity by NIR and mid-IR spectroscopy, as this statistic is commonly used to evaluate a model's predictive ability; a lower *RPD* indicates a less suitable prediction. One commonly used *RPD* threshold of 2.4 is that required for a spectroscopic method to be considered useful for rough, preliminary screening (33).

Linear regressions were calculated using mean BBR severity estimated visually as the response variable against mean BBR severity predicted by all other quantification methods: RotBot, NIR spectroscopy, mid-IR spectroscopy and qPCR. As each RotBot mean was taken from images of all bunches used in each severity group, there was only a single mean for each group. The concordance correlation coefficient (r_C) was calculated for each regression. This statistic measures how close the data are to the 1:1 line, incorporating both the accuracy and precision of the regression (19). A value of 1 would indicate complete agreement between the two methods being compared.

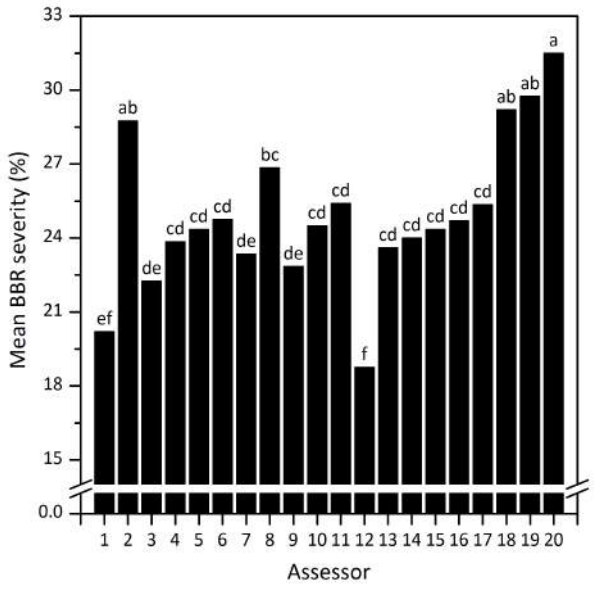

Figure 6.2 Mean botrytis bunch rot (BBR) severity from visual assessments of 20 digital images of Sauvignon blanc grape bunches with various degrees of BBR severity for 20 different assessors. Means with the same letter are not significantly different ($P < 0.05$).

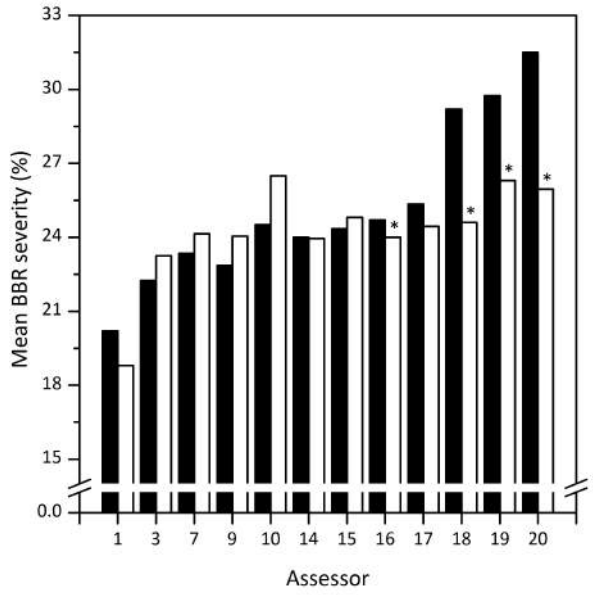

Figure 6.3 Mean botrytis bunch rot (BBR) severity from visual assessments of 20 digital images of Sauvignon blanc grape bunches with various degrees of BBR severity for 12 different assessors on two separate occasions. The time between the first (black bars) and second (white bars) assessment was 6 months. Bunch images were ordered randomly for each assessment. Second assessment means with an asterisk are significantly different from the first assessment mean for the same assessor ($P < 0.05$).

6.4. Results

6.4.1. Visual estimation

There were significant differences ($P < 0.05$) in visually estimated BBR severity among the 20 assessors (Figure 6.2), with mean BBR severity ranging from 18.1% to 31.5%. Six months later, mean BBR severity from four of 12 assessors who repeated the assessment was significantly different from their first assessment (Figure 6.3).

6.4.2. Digital image analysis (RotBot)

Hue distributions from all 26 images of Sauvignon blanc bunches identified a distinct bimodal distribution (Figure 6.4). Separating images into two groups based on BBR severity showed that for images of bunches with $\leq$ 25% BBR severity, the left peak was lower and the right peak was higher than the overall mean distribution. The opposite was true for images of bunches with $>$ 25% BBR severity. The left peak was associated with diseased tissue and the right peak associated with healthy tissue. Some pixels had hues greater than 300. The hue scale of 0–360 is circular; a hue of 5 is both 350 lower and 10 higher than 355. The hue scale was shifted to create one continuous bimodal distribution by adding 90 to all hues $\leq$ 270 and subtracting 90 from all hues $>$ 270. Hues in the range 180–270, representing blue hues, were thus shifted to the upper end of

the scale. These hues were present in the image backdrop but not in healthy or diseased tissues. From the continuous distribution of hues in the shifted hue range of 0–180, three hues were identified: $Peak_{rot}$ = the hue (shifted) for the peak identifying the part of the distribution attributable to diseased tissue; $Peak_{healthy}$ = the hue (shifted) for the peak identifying the part of the distribution attributable to healthy tissue, and Hue_{trough} = the hue (shifted) with the lowest percentage of pixels between the two peaks (Figure 6.5). These three hues were then used to determine the range of pixels corresponding to diseased tissue (Pxl_{rot}) or healthy tissue ($Pxl_{healthy}$) (Figure 6.5). Use of the three hues corresponding to the two peaks and the trough, rather than fixed hue values, allowed for potential variations in the location of the two peaks and customisation of hue ranges for each individual image.

The RotBot software loaded an image, identified the hue distribution from the pixels in that image, identified the three pixel range parameters, counted all Pxl_{rot} and $Pxl_{healthy}$ pixels and then calculated BBR severity for that image (Figure 6.6). The software also presented a stylised version of the image showing only three colours: green for healthy tissue, purple for diseased tissue and black for the background (Figure 6.7). This enabled visual confirmation that the software was adequately distinguishing diseased from healthy tissue. Images were processed at a rate of approximately 40 per min.

A significant relationship ($P < 0.05$) was found between BBR severity measured by RotBot and the mean BBR severity measured by human assessors for 26 images of

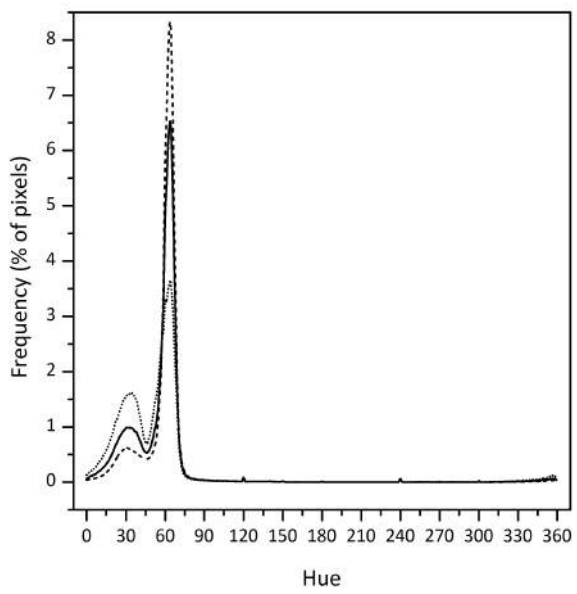

Figure 6.4 Distribution of pixels with each possible hue in 26 digital images of Sauvignon blanc grape bunches with various degrees of botrytis bunch rot (BBR) severity. Solid line = mean for all 26 images. Dashed line = mean for images of bunches with $\leq$ 25% BBR severity ($n = 16$). Dotted line = mean for bunches with $>$ 25% BBR severity ($n = 10$).

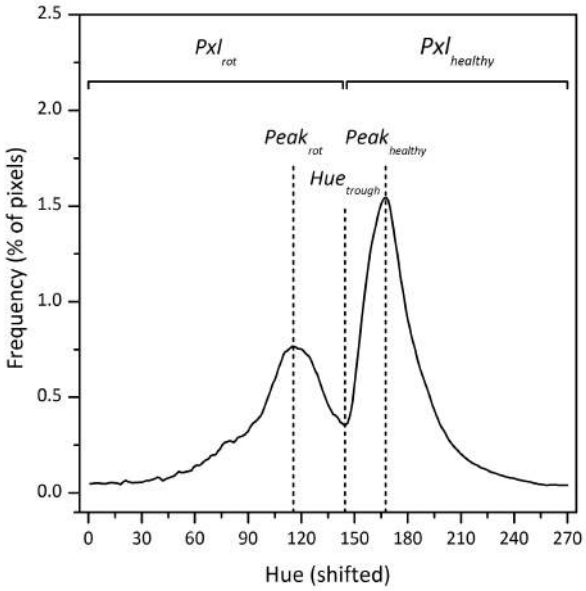

Figure 6.5 Shifted hue distribution for a digital image of a Riesling grape bunch with botrytis bunch rot (BBR) symptoms showing parameters identified by RotBot ($Peak_{rot}$, $Peak_{healthy}$ and Hue_{trough}) that were used to determine whether pixels were attributable to diseased (Pxl_{rot}) or healthy ($Pxl_{healthy}$) tissue. The x-axis shows hue values shifted by adding 90 to all values $\leq$ 270 and subtracting 90 from all values $>$ 270. The hue range of 180–270 (shifted range of 270–360) was likely to be associated with the blue background and is therefore not shown.

Sauvignon blanc bunches with various degrees of BBR severity (Figure 6.8). RotBot tended to measure lower BBR severity than the assessors' estimates.

Use of one or both sides of Riesling bunches for image analysis did not affect mean BBR severity calculated by RotBot. When the original image order was used to designate which of the two images for each bunch was the first and which the second side, there was a significant difference ($P < 0.05$) between mean BBR severity measured by RotBot using just the first or second sides; however, mean BBR severity using images of both sides was not significantly different ($P > 0.05$) from the mean for either side alone (Table 6.2).

Table 6.2. Mean botrytis bunch rot (BBR) severity (%) for 66 Riesling grape bunches collected in 2013 measured by RotBot using images of the first side, the second side, or both sides, compared with the mean visual estimate from the original bunches (one side only). The two images captured for each bunch were designated as the first side and the second side using the original order in which the images were captured and also by randomly assigning the two images to either the first or second side. Means with the same letter within the same column are not significantly different ($P < 0.05$).

Images Used	Original order	Randomly assigned order
First side	31.3 b	29.3 b
Second side	26.6 c	28.6 b
Both sides	29.0 bc	29.0 b
Visual estimate	35.0 a	35.0 a

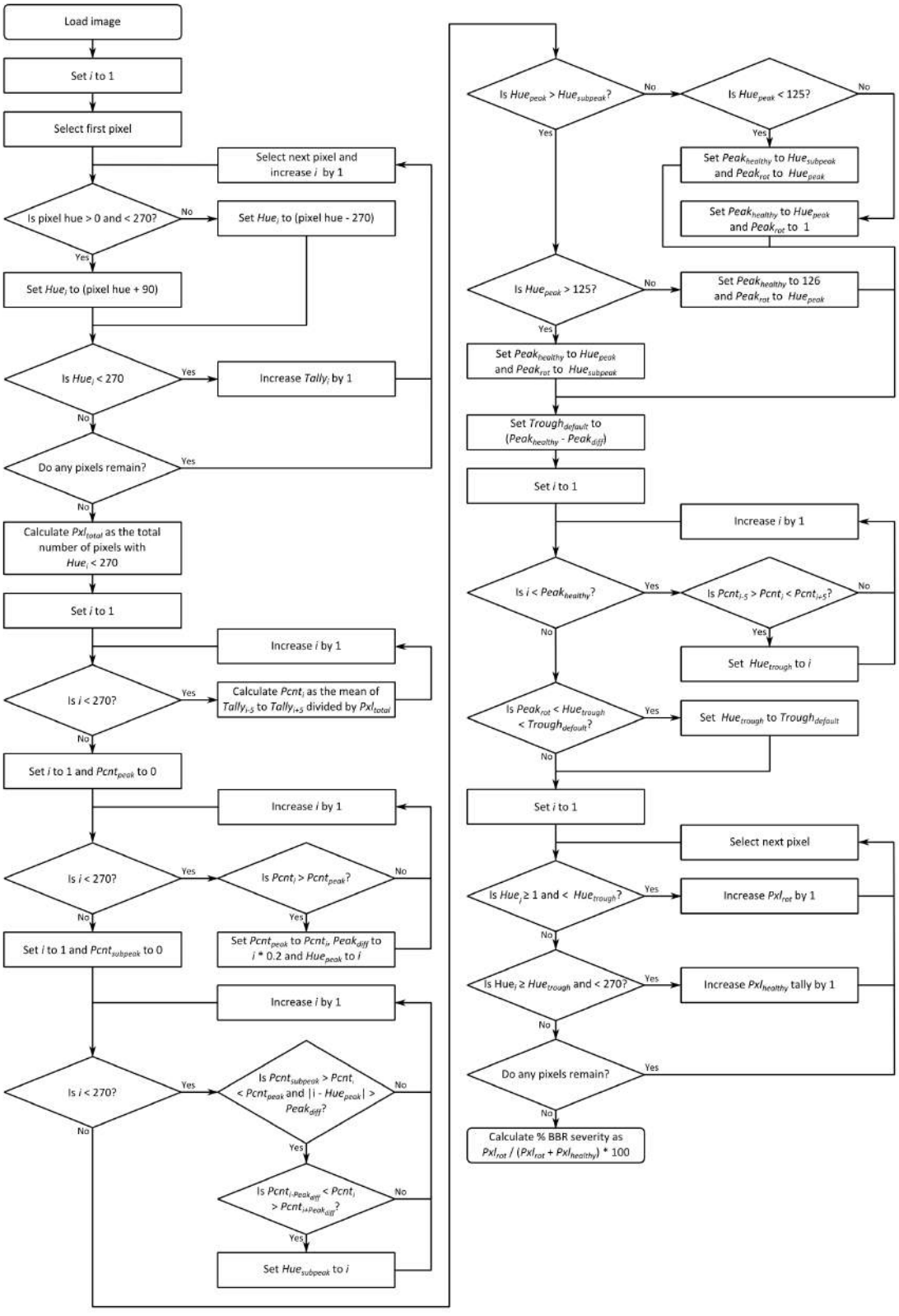

Figure 6.6. RotBot flowchart showing computational steps in the calculation of botrytis bunch rot (BBR) severity from a digital image of a grape bunch.

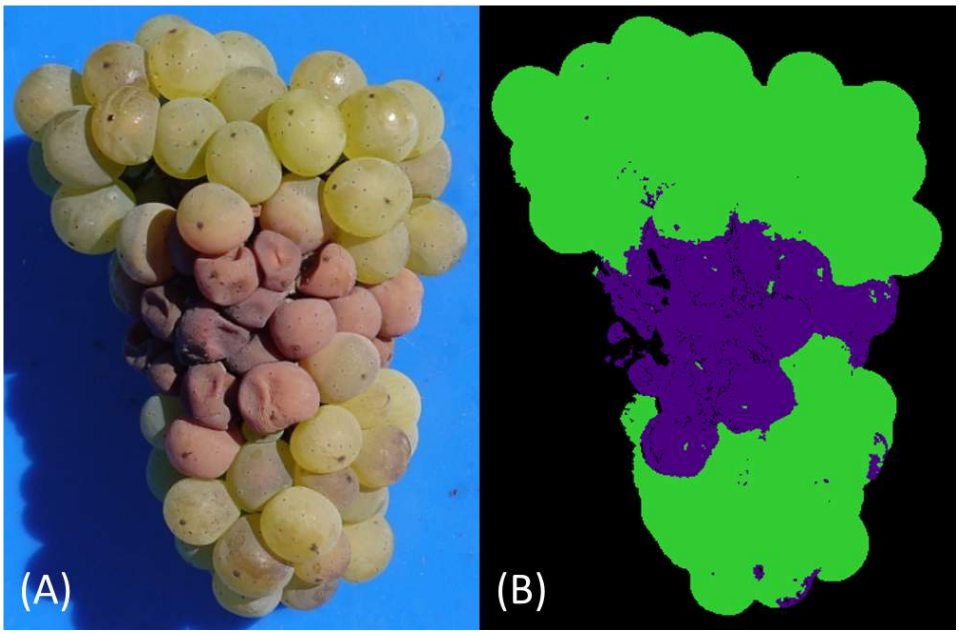

Figure 6.7. Botrytis bunch rot (BBR) symptoms on a Riesling grape bunch (A) before and (B) after analysis by RotBot software: pixels were designated as either healthy (green), diseased (purple) or background (black). RotBot estimated 25% BBR severity for this image.

There was no significant difference ($P < 0.05$) between the first and second side when side was randomly assigned. Mean BBR severity measured by RotBot was significantly lower ($P < 0.05$) than the visual estimate in all comparisons.

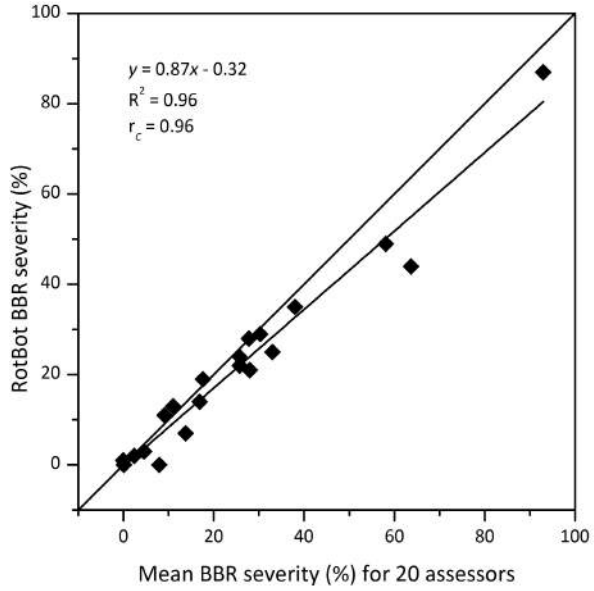

Figure 6.8 Linear regression of botrytis bunch rot (BBR) severity measured by RotBot versus mean BBR severity measured by 20 assessors for 20 digital images of Sauvignon blanc grape bunches. Dotted line represents $y = x$.

6.4.3. Quantitative PCR

There was a strong log-linear relationship between Cq value and *B. cinerea* or *V. vinifera* DNA quantity for qPCRs containing either *B. cinerea* or *V. vinifera* DNA and either Bc3 or Res primers ($P < 0.001$, $R^2 = 0.99$; Figure 6.9). Cq values from reactions with Res primers with *V. vinifera* DNA were lower for a given quantity of *V. vinifera* DNA than values from the Bc3 primers amplifying the equivalent quantity of *B. cinerea* DNA.

For duplex assays with Res and Bc3 primers, Cq values for reactions where 0.001 ng of *B. cinerea* DNA was used were significantly lower ($P < 0.05$) when *V. vinifera* was added to the reaction mix than when sterile water was added (Figure 6.10); this was not the case when 0.01–10 ng of *B. cinerea* DNA was used. The amount of *V. vinifera* DNA amplified was similar for different quantities of *B. cinerea* DNA in reactions containing both types of DNA (Figure 6.10).

A significant, positive logarithmic relationship ($P < 0.05$) was observed between PC from duplex qPCR and BBR severity determined by weight of rotten berries for the 2012 manufactured samples (Figure 6.11). The equation from this regression was rearranged and used to calculate BBR severity for the 2013 samples after qPCR:

$$f(x) = e^{((PC-1.06)/0.15)} \tag{6.1}$$

where x = predicted BBR severity (%).

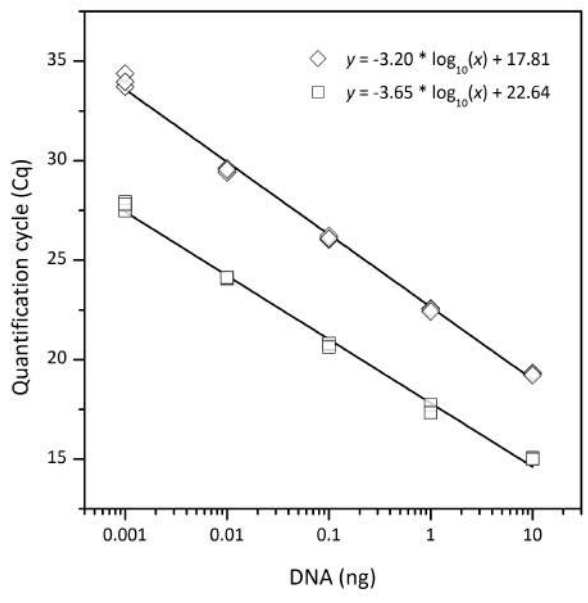

Figure 6.9 Standard curves showing quantification cycle (Cq) in relation to amount of DNA in each quantitative PCR (qPCR) run containing either *Botrytis cinerea* or *Vitis vinifera* DNA. One curve represents quantification of *B. cinerea* DNA with Bc3 primers (diamonds; $R^2 = 0.99$; $P < 0.001$) and the other quantification of *V. vinifera* DNA with Res primers (squares; $R^2 = 0.99$; $P < 0.001$). DNA origin is described in Table 6.1.

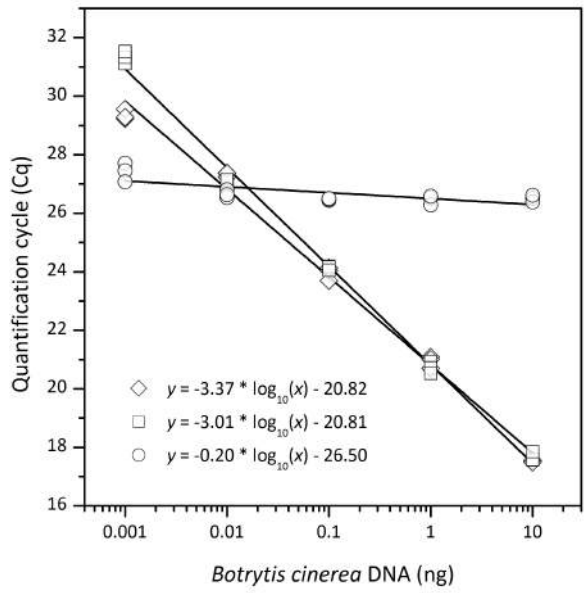

Figure 6.10 Linear regressions of quantification cycle (Cq) resulting from duplex quantitative PCR (qPCR) versus the amount of *Botrytis cinerea* DNA added to the reaction mix in either 10 ng *Vitis vinifera* DNA (diamonds; $R^2 = 0.99$; $P < 0.001$) or water (squares; $R^2 = 0.99$; $P < 0.001$). Cq for Res primers with Res_P probe from reactions with 10 ng *V. vinifera* DNA is also shown (circles; $R^2 = 0.50$; $P = 0.002$). DNA origin is described in Table 6.1.

6.4.4. Infrared spectroscopy

Inspection of spectral plots of absorbance data within the mid-IR spectral region of 8760–9520 nm revealed clear separation of the 0-25% BBR severity groups (2013 Riesling bunches) and the higher severity groups for both raw and pre-processed data (Figure 6.12b,c). Such separation was not observed for the NIR absorbance spectra (Figure 6.12a,c).

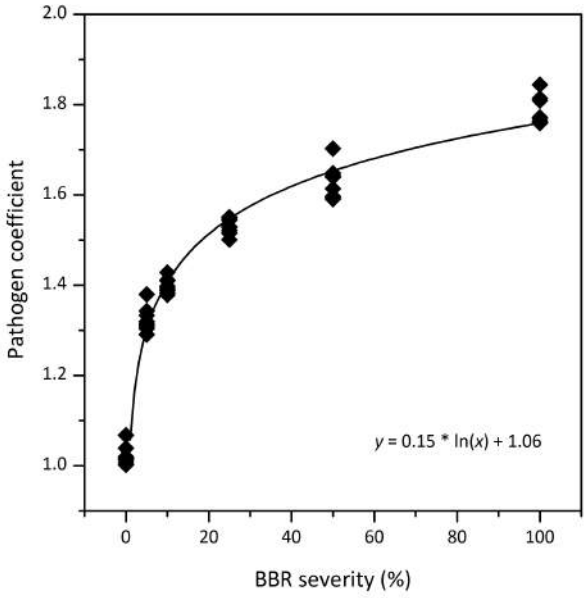

Figure 6.11 Logarithmic regression of pathogen coefficient (PC) from duplex quantitative PCR (qPCR) versus botrytis bunch rot (BBR) severity determined for the 2012 manufactured samples of Riesling grape berries ($R^2 = 0.96$; $P < 0.001$).

Table 6.3. Covariance analysis showing estimated percentage variance for three levels of replication used in obtaining pathogen coefficient (PC) values from homogenised grape berries via quantitative qPCR (qPCR). Replication was nested in the order of: container of berries, ground tissue sub-sample, and sub-sample of DNA solution. 'Residual' includes all variation not attributed to replication of berries or ground tissue; the majority of this variation can be attributed to DNA replication. The percentage variance estimate shown is only for the variance additional to that attributed to the parameter nested below it.

Covariance Parameter	Estimated % variance	P-value
Berries	0.0	-
Ground tissue	72.9	<0.001
Residual (including DNA)	27.1	<0.001

6.4.5. Comparison to visual estimation

A significant relationship ($P < 0.05$) was found between BBR severity predicted by RotBot and BBR severity estimated visually for the 2013 samples (Figure 6.13a). RotBot measured lower BBR severity than visual estimation; however, the r_C was the highest of all methods compared.

There was a significant relationship ($P < 0.05$) between BBR severity predicted by qPCR and BBR severity estimated visually (Figure 6.13b). Data points were scattered above and below the 1:1 relationship; however, 18% of the variance in the linear prediction of mean BBR severity was not accounted for by BBR severity estimated visually. There was substantial variation in predicted BBR severity among replicates for the higher severity groups, particularly at 50% severity. Covariance analysis found that the majority of this variation was from subsamples of ground tissue prior to DNA extraction, with some variation arising from sub-sampling of the DNA solution prior to qPCR (Table 6.3).

A significant relationship ($P < 0.05$) was found between BBR severity predicted by both NIR (Figure 6.13c) and mid-IR (Figure 6.13d) spectroscopy and BBR severity estimated visually for Riesling bunches sampled in 2013. NIR spectroscopy gave an *RPD* of 2.5 and mid-IR spectroscopy gave an *RPD* of 2.0. Both spectroscopic methods measured lower BBR severity than visual estimation. The majority of severity values predicted by mid-IR for the 0-25% groups were below 0%. There was no significant difference ($P > 0.05$) between severity groups 5, 10 and 25% in mean BBR severity predicted by either NIR or mid-IR spectroscopy.

6.5. Discussion

This study revealed that assessors show significant variation in visual estimation of BBR severity, even when they have previous experience with BBR assessments. The majority of assessors were consistent when asked to repeat assessments months later. Therefore, it may be possible to quantify an assessor's bias using software such as the Bunch Rot

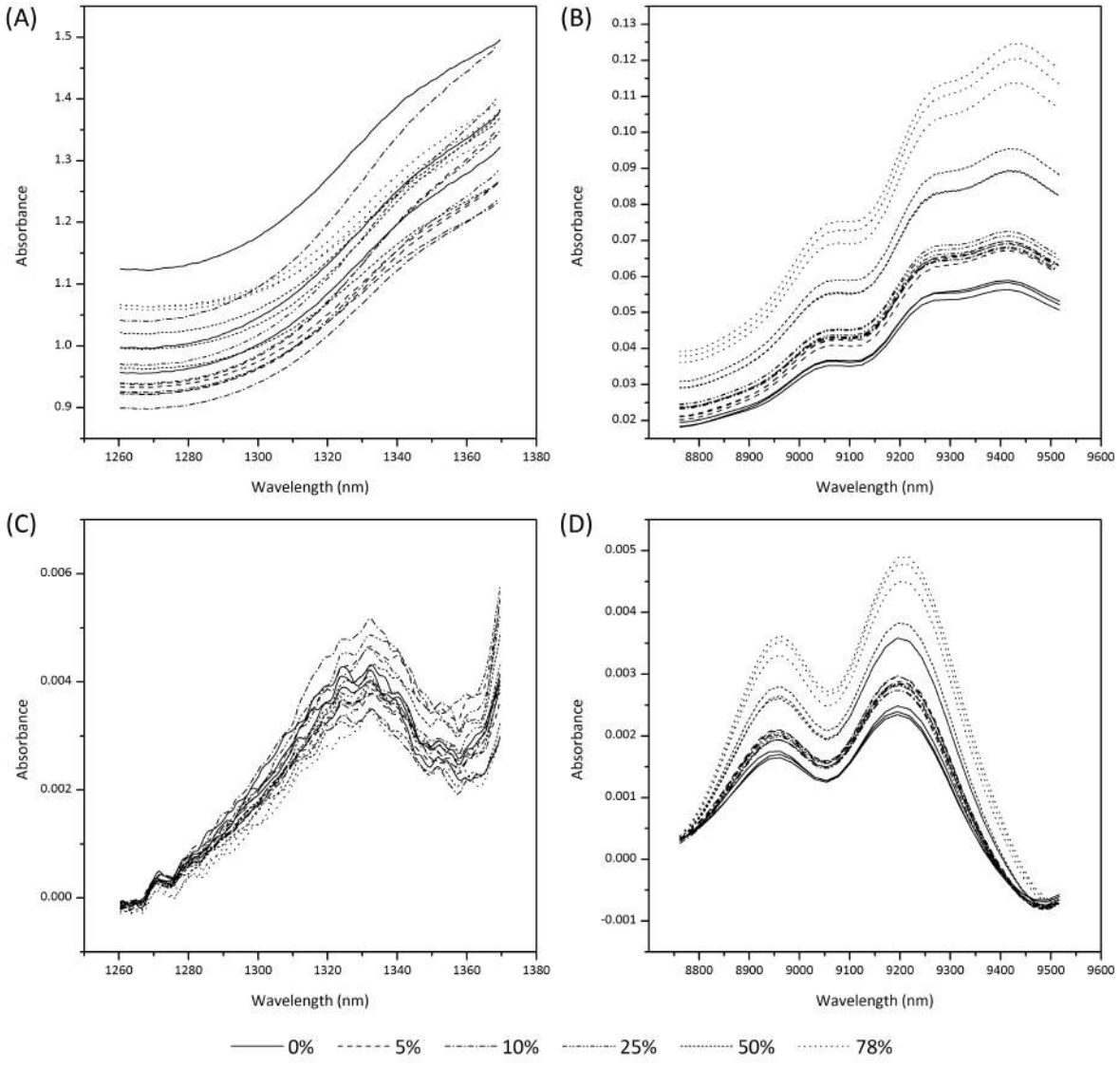

Figure 6.12. Absorbance spectra for all replicates in each botrytis bunch rot (BBR) severity group of 2013 Riesling grape samples in the near-infrared (NIR) spectral range of 1260–1370 nm and the mid-infrared (mid-IR) spectral range of 8760–9520 nm. (A) Raw NIR data. (B) Raw mid-IR data. (C) NIR data pre-processed using the Savitzky-Golay derivative. (D) Mid-IR data pre-processed using the Savitzky-Golay derivative.

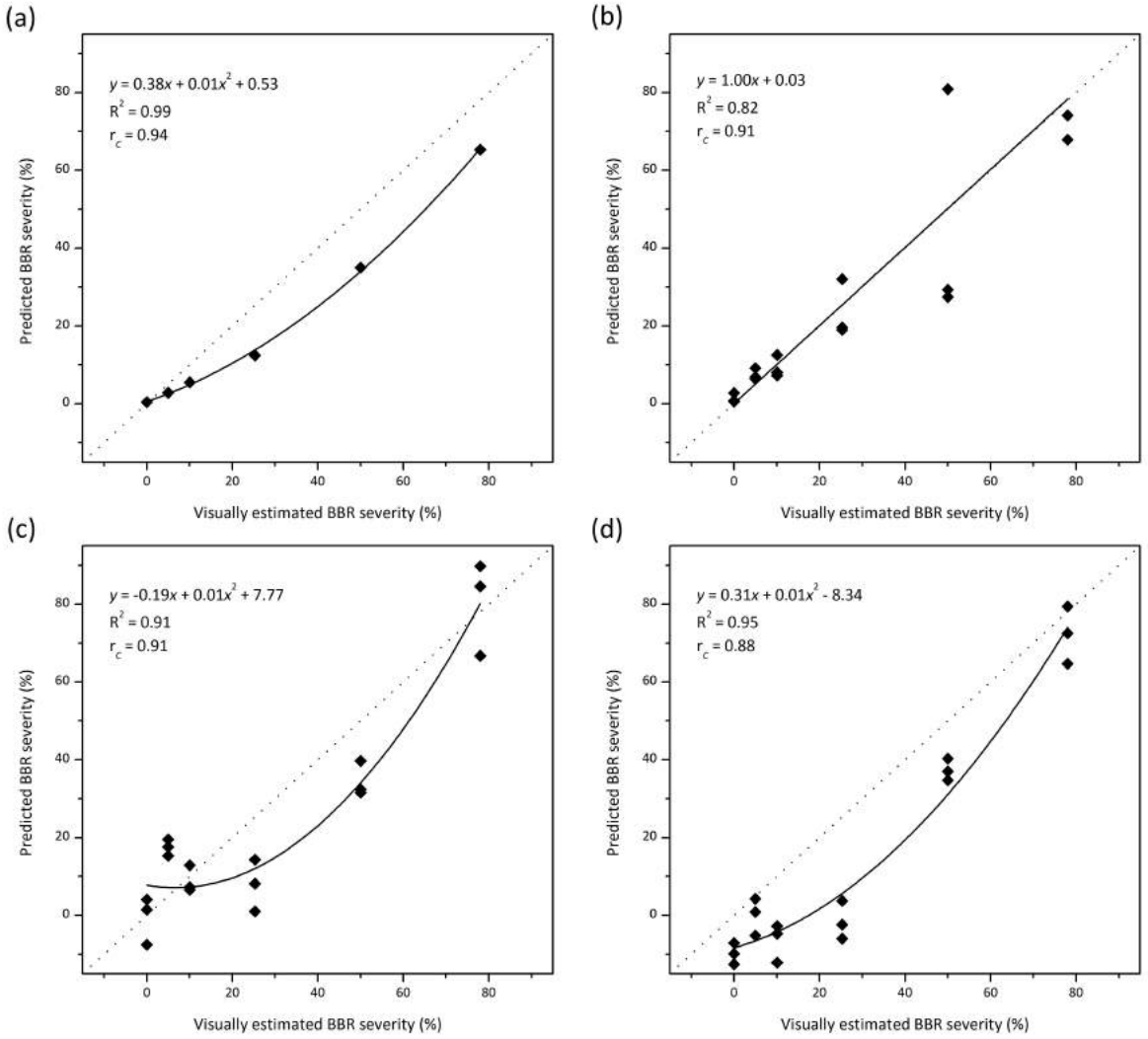

Figure 6.13. Regressions of mean botrytis bunch rot (BBR) severity in grapes estimated visually against mean BBR severity predicted by: (A) RotBot, (B) quantitative PCR (qPCR), (C) near-infrared spectroscopy (NIR) and (D) mid-infrared spectroscopy (mid-IR). Dotted line represents $y = x$. All regressions were significant ($P < 0.001$).

Assessment Trainer (17), if the bias is consistent. An equation could then be generated to correct assessment data for each assessor.

Digital image analysis had the advantage of eliminating assessor bias by using the distribution of pixel hue to distinguish diseased from healthy tissue. Hue was used to minimise the effect of light intensity changes during image capture, although the effect of light conditions on the ability of RotBot to measure BBR severity was not investigated in this study. The hue ranges used to identify diseased tissue were calculated for each image, thus accounting for variations in hue distributions among images.

The difference between BBR severity estimated visually and by RotBot might be explained by the assessor increasing their estimate to compensate for berry shrivelling or by including bunch rot symptoms caused by other diseases that are not detected by RotBot. RotBot might also misclassify pixels at the edges of hue ranges or for very minor symptoms.

The significant difference in mean BBR severity between sides of the bunch may have occurred because the more heavily infected side was subconsciously photographed first. To prevent skewed results, bunches should be placed randomly in the tray, or images of both sides used for estimation of BBR severity. Photographing bunches on the vine would remove any bias from the assessor, as they would always photograph the side facing outwards. However, it might introduce a new bias if inner and outer sides differed in severity as a result of environmental factors, such as UV-light exposure (29) or canopy microclimate (11).

Use of spectroscopy for quantification of BBR severity would allow the addition of BBR assessment to existing winery analyses without requiring additional sampling. NIR and mid-IR spectroscopy did not predict BBR severity as precisely as RotBot; however, there was still a significant relationship with visual estimates. The *RPD* for NIR spectroscopy method suggests that it is only suitable for rough screening, while the current mid-IR spectroscopy method would not be recommended based on the *RPD*. The reason mid-IR spectroscopy had a lower *RPD* was possibly because the method predicted many negative values, while NIR spectroscopy only predicted a single negative value. Both methods appeared to be more accurate at predicting the 50% and 78% severity groups. There was considerable non-linearity in the relationship for the lower severity groups. This observation is not surprising when the BBR severity of individual bunches used to made up each severity group is considered. For example, the 5% group consisted mostly of bunches with 1–5% severity and one bunch with 20% severity. If the visual estimation of that one bunch was under-estimated substantially, the mean severity for the group may be inaccurate. There may have been more consistent predictions if individual bunch severities were clustered around the mean or if more bunches were pooled to create each group. Alternatively, the highly infected fruit may show different fungal metabolite and secondary fruit damage profiles to low infection fruit; therefore, spectral fingerprints would differ, necessitating separate calibrations for high and low infection levels. The thresholds of BBR severity for crop rejection are nearly always set below 10%. Since neither NIR nor mid-IR spectroscopy was able to separate severity groups of 5, 10 and

25%, both methods would need to be further calibrated at low severities to be considered useful alternatives to visual estimation.

In contrast to the spectroscopic methods, qPCR gave high accuracy at low severities and lower accuracy at higher severities. Even with substantial variation among sample results in the 25% and 50% severity groups, the linear fit was the closest to 1:1 of all the methods tested. Future research might explore the use of PC as the quantification variable, rather than using its empirical relationship with visual estimation to calculate BBR severity. If so, qPCR might provide a highly sensitive and objective measure of BBR severity in the range of practical interest (0–10%). Nevertheless, sample preparation for qPCR, including tissue grinding and DNA extraction, is extremely labour intensive and requires specialised equipment. Therefore, qPCR is only suitable as a research tool and not for routine use in the vineyard.

None of the quantificaiton methods were tested for their ability to distinguish bunch rots caused by *B. cinerea* from those caused by other organisms.

A reference method is essential for calibration of any analytical technique. This study used visual estimation as the reference method as it is widely used and understood, but it was prone to assessor variability and bias. The PC from qPCR has potential to be the reference method, particularly if this variable can be related to subsequent wine quality and style. Spectroscopic models calibrated using the PC as a reference method would probably be more objective and potentially more accurate. RotBot is also objective, as long as the side of the bunch photographed is randomly selected, or both sides are photographed. Therefore, RotBot could also be used as a reference in the calibration of the spectroscopic methods. Use of any alternative to visual estimation would depend on whether the goal was to find an alternative that predicted BBR severity in a way that was directly comparable to visually estimated BBR severity, or whether the goal was to find an alternative method for the quantification of BBR that removed subjectivity regardless of the variable used. This distinction will be an important consideration should one of the methods discussed here be developed into a standardised tool for the quantification of BBR.

6.6. Acknowledgements

This work was supported by The New Zealand Institute for Plant & Food Research Limited and the Tasmanian Institute of Agriculture (TIA), University of Tasmania. We

thank Justin Direen and Michele Buntain (TIA) for assistance with sample collection. We also thank our vineyard co-operators from Brown Brothers and John McKay (DNAture Ltd, Gisborne, New Zealand) for assistance in optimising probes.

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7

General Discussion

The purpose of this study was to clarify aspects of the pathogenic interaction between *Botrytis cinerea* Pers. and *Vitis vinifera* L. grapes, focusing on current gaps in the understanding of the disease cycle and epidemic development botrytis bunch rot (BBR). The main areas of research included the relative importance of different grape vine growth stages in the establishment of *B. cinerea* infection (Chapter 3), growth within grape berries during the latent period (Chapter 4), and how to best quantify BBR once symptoms are expressed (Chapter 5 and Chapter 6).

Infections occurring at each of the growth stages investigated (flowering, pre-bunch closure (PBC) and veraison) led to the establishment of latent infections that were able to persist through to harvest. Flowering had previously been considered to be a particularly important growth stage for infection, although previous studies on *B. cinerea* infection, particularly those involving inoculations, have focussed primarily on the flowering or post-veraison period and have not simultaneously investigated intermediate growth stages for direct comparison (Keller *et al.*, 2003, Nair *et al.*, 1995, Pezet *et al.*, 2003). The findings from this study suggest that no single phenological stage is more important than any other for *B. cinerea* infections that can lead to BBR development. While BBR severity developed more rapidly for bunches inoculated at veraison in the 2012/13

field trial (Figure 3.8), the effect of inoculation time on BBR severity at harvest was not statistically different when data from all trials were combined (Figure 3.9). As *B. cinerea* conidia can be present in the vineyard air spora at all times during the season (Rodríguez-Rajo *et al.*, 2010), infections are potentially occurring at all phenological stages. This finding could have an impact on BBR management in climates and seasons conducive to season-long infection by *B. cinerea*, when an effective dose of protective fungicide might be needed from flowering to harvest. As fungicide residue limits continue to be reduced, certain fungicides may be limited to flowering applications (Chapter 2.3.6.1). More emphasis must now be placed on the development and integration of effective control measures throughout the season, such as canopy management (Chapter 2.3.6.3) or biological control agents (Chapter 2.3.6.2), to combat a potentially continuous risk of infection.

There are two potential pathways by which BBR can develop following infections at flowering: latent infections and infected floral parts becoming trapped in bunches ('bunch trash') and acting as sources of inoculum as the berries mature (Elmer & Michailides, 2004). As latent infections had established within one week of inoculation in the glasshouse trial (Figure 3.6), which is a relatively short time period, it is likely these infections resulted directly from the inoculations, rather than from infection occurring later via infected bunch trash. It is unclear whether latent infections found in berries that were inoculated at flowering and collected at PBC and veraison were established at flowering or later in the season as the result of infected bunch trash or polycyclic infection. Microscopy showed that flowers could become heavily colonised by *B. cinerea* hyphae within 5 d (Figure 4.7), supporting the bunch trash hypothesis for subsequent infection. Confocal microscopy did not identify the location of infections in inoculated flowers and berries due to loss of green fluorescent protein (GFP) fluorescence. Therefore, it remains unclear whether latent infection or bunch trash is the predominant means by which flower infection leads to BBR symptoms later in the season.

Microscopy did not reveal where infection was occurring or whether latent infections were present in inoculated berries at PBC and veraison. The only clear infection sites were wounds on veraison berries that had split (Figure 4.11). Very little hyphal growth was observed on *V. vinifera* Sauvignon blanc berries at PBC or on berries at veraison without wounding, whereas hyphal growth was extensive in wounded *V. vinifera* Crimson Seedless and Sauvignon blanc berries at veraison (Figure 4.5 and Figure 4.11). No spore germination was observed on non-wounded Crimson Seedless berries and germinated spores on wounded berries were only observed close to the edges of wounds (Figure 4.3).

Wounding has previously been found to increase the infection rate of *B. cinerea* on grape berries (Mundy & Beresford, 2007, Nair *et al.*, 1988). Nutrients exuded from fruit have been found to increase *B. cinerea* spore germination (Fourie & Holz, 1998, Kosuge & Hewitt, 1964); wounding may increase the concentration of these nutrients on the berry surface in the surrounding area and therefore increase percentage germination. Post-veraison, maturing grape berries are easily wounded through berry splitting and damage from external sources such as birds and insects (Coley-Smith *et al.*, 1980), whereas at PBC, berry skins generally have greater integrity and are less prone to wounding (Commenil *et al.*, 1997). It has previously been found that immature berries appear less susceptible to *B. cinerea* infection than mature berries (McClellan & Hewitt, 1973, Nair *et al.*, 1988). Therefore, it is unclear how infection at PBC could lead to a degree of BBR severity equivalent to infection at later stages, as demonstrated by the inoculation trials. Further study using microscopy needs to be done on berries at PBC to determine whether *B. cinerea* is penetrating the skin directly or opportunistically via wounds or cracks in the skin.

The percentage of latent infections fluctuated over time in some treatments in the glasshouse trial (Figure 3.6). This fluctuation may have been due to the overnight freezing and incubation technique (ONFIT), as factors such as variation in laboratory conditions during incubation and the effectiveness of surface sterilisation could have affected the ONFIT results at each growth stage. Surface sterilisation used with the ONFIT may not be completely effective at killing all microorganisms on the berry surface, or may kill some of the latent *B. cinerea* infections. Therefore, differences between treatments observed for the same ONFIT are more meaningful than comparisons of results from the ONFIT carried out at different times. There may have been mortality of latent infections in some bunches, as the strain frequency has been found to vary between flowering and harvest in commercial vineyards across New Zealand (Johnston *et al.*, 2013). Additionally, it has been found that strains collected at flowering with high pathogenicity do not necessarily persist through to harvest (Johnston *et al.*, 2013). It is possible that some strains are more pathogenic but less fit, meaning they will readily infect but cannot survive as latent infections long enough to produce symptoms.

The nature of *in planta* fungal growth following infection and prior to symptom expression remains unclear. Viret *et al.* (2004) studied the infection of grape flowers by *B. cinerea* and *in planta* growth and found that floral parts were heavily colonised after 72 h; however, this study did not make a link between this fungal colonisation and BBR symptoms at harvest. McNicol and Williamson (1989) conducted a similar study in

blackcurrant flowers and observed the *in planta* growth of *B. cinerea* for a longer period of time (28 days). They observed hyphal growth in flowers that showed no external symptoms of infection. It was hoped in this study to observe latent infection in both grape flowers and berries from infection through to symptom expression. Unfortunately, sample fixation required by regulations on the use of genetically modified organisms (GMOs) resulted in loss of GFP fluorescence. Therefore, the work did not characterise fungal growth as extensively as intended. It was not possible to observe whether or not latent infections had established in Sauvignon blanc berries as auto-fluorescence from the host cells masked the fluorescence from GFP-expressing hyphae beneath the skin surface.

Flowers were readily colonised by *B. cinerea* within a few days (Figure 4.7), as previously observed by Viret et al. (2004). However, latent infections were found in berries that had been inoculated at flowering. If flowers were as readily colonised on the vine as the detached flowers were in this study, they would probably rot and abscise before developing into a berry (Ngugi & Scherm, 2006), which would prevent latent infections from establishing at this growth stage. This suggests there is a mechanism by which the living host prevents such extensive hyphal growth. Alternatively, the high concentration of spores used in the inoculations of detached inflorescences may have resulted in an artificially high amount of hyphal growth. Although spore density was found here to be negatively correlated with percentage spore germination (Figure A.2), its effect on the resulting hyphal growth rate was not investigated.

Negligible hyphal growth was observed in PBC berries (Figure 4.10). It is possible that the lack of hyphal growth seen in PBC berries was due to the berries being less susceptible to infection (Hill *et al.*, 1981). However, inoculations at this phenological stage led to the establishment of latent infections (Figure 3.7).

The majority of hyphal growth in mature Sauvignon blanc and Crimson Seedless berries was observed between the cuticle and epidermis (Figure 4.6 and Figure 4.5). No hyphae could be traced back to germinating spores, so no conclusions on how *B. cinerea* penetrated host tissue can be drawn. It is possible that growth originated from within wounds and that the cuticle was not penetrated in this instance. Asymptomatic areas of Crimson Seedless berries adjacent to areas with aerial hyphae contained many branched hyphae directly beneath the cuticle, but very few hyphae were seen penetrating below the epidermis. This growth was also observed in Sauvignon blanc berries at veraison. Previous studies have used the location of aerial hyphal growth from incubated berries as evidence of infection sites, with one of the predominant infection sites suggested as being the receptacle end (Holz *et al.*, 2003). An alternative hypothesis is that the natural gap

between the receptacle and the berry skin provides a suitable site for the fungus to grow out of the berry after hyphae have been proliferating throughout the berry between the cuticle and the epidermis. Sealing detached berries in paraffin wax and wounding them at various locations prior to incubation may elucidate whether the receptacle end of the berry is a site of infection or simply the most readily available exit point for the fungus.

Extensive hyphal growth between the cuticle and epidermis may explain the different symptoms caused by *B. cinerea*. Hyphae may continue to grow under the cuticle in mature berries until conditions are suitable for the production of one of three possible symptoms: BBR, slip skin or noble rot. When symptoms develop following latent infection, berry and external conditions most likely dictate which symptoms will occur. BBR symptoms may be influenced by certain conditions such as favourable berry sugar content (Mundy & Beresford, 2007) and weather variables (Broome *et al.*, 1995, Kim *et al.*, 2007). Under these conditions, the pulp may become more susceptible to infection, possibly through changes in osmotic potential (Harris, 1981), allowing hyphae to penetrate deeper and rot the berry. The fungus may also produce infection cushion-like structures (Backhouse & Willetts, 1987), as observed with confocal microscopy (Figure 4.13), to penetrate out of the cuticle and produce other BBR symptoms such as aerial hyphae and conidiophores. Alternatively, berry splitting or other berry damage may allow the fungus to grow onto the surface of the berries to produce BBR symptoms. Slip skin symptoms have been known about for some time (Hewitt, 1974, Nelson, 1956); however, dedicated research has only recently begun to investigate the cause of these symptoms (Beresford *et al.*, 2013). Conditions suitable for slip skin symptoms may restrict hyphae to subcutaneous growth, degrading the epidermis and leaving the pulp relatively intact, causing the skin to slide off the pulp when pressure is applied. Noble rot symptoms may be favoured by warm, windy days that facilitate water evaporation, increasing osmotic potential, which limits fungal growth, and reducing berry splitting (Vannini & Chilosi, 2013). Reducing splitting is said to reduce the chance of *B. cinerea* infection by reducing potential infection sites; an alternative hypothesis is that less berry splitting increases the likelihood of the hyphae remaining beneath the cuticle and not producing BBR symptoms. Comparing the skins and pulp of berries with different *B. cinerea* symptoms, and making note of the weather conditions leading to those symptoms, may shed light on the different conditions leading to these different symptoms.

Since it appears that infection can occur throughout the season, it is possible that a polycyclic epidemic could occur, which involves secondary infection conidia or hyphae produced from symptomatic bunches. The time between inoculation at veraison and

symptom expression was found to be considerably shorter than the length of time between symptom expression and harvest (9 days and 15 days, respectively; Figure 3.8). An experiment in which symptomatic bunches were removed from vines in the field as symptoms appeared on them during ripening (Appendix B) showed that bunch removal did not slow the increase in BBR incidence or significantly reduce BBR incidence at harvest compared with a control where no bunches were removed. If secondary infection had occurred in this instance, it would have had to have occurred prior to bunch removal or from inoculum produced by bunches other than those in the immediate vicinity. Conclusions from this trial are to be made with caution as the methodology was flawed. In order to better investigate the potential for secondary infection to influence BBR epidemics, bunches would need to be removed prior to symptom expression and from nearby rows and spore concentration in the vineyard air spora should be monitored.

The current standard method for quantification of BBR symptoms is visual estimation, which can be time consuming and prone to assessor bias. In this study, visual estimation was evaluated and compared to four alternative quantification methods: near-infrared (NIR) spectroscopy and mid-infrared (mid-IR) spectroscopy, quantitative PCR (qPCR) and digital image analysis (RotBot).

Visual estimation was found to produce significantly different BBR severities among assessors (Figure 6.2), but not for the same assessor over time (Figure 6.3). The error in this method can be reduced through the use of severity assessment keys and training software (Hill *et al.*, 2010). However, the method is labour intensive and there is potential for other diseases to mask BBR symptoms, as seen in the 2010/11 field trial. The latter may only be a disadvantage for researchers, as grapes infected with other diseases in commercial vineyards are likely to still be a concern for wine makers and require quantification (Godden, 2000).

NIR and mid-IR spectroscopy methods for the quantification of BBR were developed (Chapter 5); both methods showed potential as alternatives to visual estimation (Figure 6.13c,d). However, further calibration of the models is required as the accuracy of the predictions made using either spectral range was too low at mean severities of $<$ 25%, which are of most concern to grape growers and winemakers. The accuracy of the methods could be improved if more emphasis were placed on lower severities in future calibrations. BBR severities $>$ 20% are rarely seen in commercial vineyards. Even with artificial inoculation, the highest mean BBR severity for any treatment in the 2012/13 field trial was only 12.7%. Future calibrations of the spectroscopy models using samples with mean severities of $<$ 20%, separated into groups at 1% intervals,

may produce quantification methods with more commercial value. The resulting models could potentially have an accuracy of 1% and this degree of accuracy would be necessary to quantify BBR severity around the 3–5% threshold. Initial validation of any newly calibrated spectroscopy models should use the same severity categories as those used in the calibrations, followed by validation using randomly collected samples that represent actual severity in each vineyard sampled. The newly calibrated models should also be tested on multiple varieties to ensure it is not a variety-specific detection and on samples infected with other diseases to ensure the method is specific to BBR.

The RotBot software was written to quantify BBR severity by analysing images of white grape bunches with BBR symptoms and differentiating diseased and healthy tissue using the hue of each pixel (Chapter 6). The predictions of BBR severity made by this software were highly correlated with visual estimation (Figure 6.13a). RotBot only assesses visible symptoms; however, this is also true for visual estimation so it does not affect RotBot's suitability as an alternative method. This software could easily be developed as a web-based or smartphone application. The advantage of a web-based application is that vineyard managers and grape growers around the world could use the method, so long as they had a digital camera and an internet connection. The advantage of a smartphone application would be that photographs could be taken and assessments made rapidly at the site where grape bunches are sampled. Before software can be released on any platform, the method needs to be standardised, such as the use of a blue background during photography to allow the software to distinguish each bunch. The use of such a background may be difficult when bunches remain on the vine. The software could be developed to automatically distinguish the bunch using an algorithm such as that developed by Nuske *et al.* (2011), which selects grape berries in images of whole vines using radial symmetry.

The qPCR method, modified from that published by Saito *et al.* (2013), was the most accurate method tested (Figure 6.13b). The linear fit of predicted BBR severity versus actual was almost indistinguishable from a 1:1 fit, despite a lack of precision in the higher severity categories. The main disadvantage of qPCR is the time required for sample preparation. As the primers used in the qPCR method were highly specific to *B. cinerea* DNA (Suarez *et al.*, 2005), the time required for the qPCR method may be justifiable in a research setting where accuracy and specificity are required. Increasing the number of subsamples of ground tissue taken would be recommended to compensate for lower precision. Quantitative PCR also has the potential to quantify other disease-causing organisms within the same bunch sample, so long as primers specific to those organisms

were available and that they could be used in a duplex PCR with the *V. vinifera*-specific primers.

The samples used to compare quantification methods were also tested using two commercially available laccase assays, Dolmar Laccase Detection Kit (Dolmar, Haro, Spain) and Botrytest (Laffort, Bordeaux, France), and one 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) laccase assay (Appendix C). Significant relationships were found between BBR severity and laccase activity from all three assays (Figure C.1); however, the two highest severity categories were not differentiated by either of the commerically available assays or by the ABTS laccase assay without diluting the samples. The ABTS laccase assay gave the highest coefficient of determination (R^2= 0.99) with sample dilution. Although this method looks promising, the samples were pooled and not replicated. Therefore, no conclusion could be made about the suitability of the ABTS laccase assay as an alternative quantification method.

While all methods tested showed some potential as alternative methods for the quantification of BBR symptoms, only some of them would be useful for quantifying latent infections earlier in the season. Visual estimation and RotBot would not be suitable for this purpose as they only measure visible BBR symptoms. Laccase assays use juice from mature berries and are also not suitable. NIR and mid-IR spectroscopy may have the ability to detect latent infections, but it is not known exactly what these methods are quantifying. Model calibration using immature berries or flowers with known percentages of latent infections would be required to determine whether spectroscopic methods could quantify amount of latent infection. Quantitative PCR has the potential to detect and quantify latent infections as this method quantifies *B. cinerea* DNA, which is present whether symptoms are present or not.

Conclusions and future research

The following main conclusions were drawn as a result of this study, organised according to stage in the disease cycle:

- Infection
  - Infection of grape inflorescences or bunches by *B. cinerea* can potentially occur at any phenological stage from flowering to harvest.
  - There appears to be no particular growth stage at which infection leads to significantly greater BBR severity at harvest.
- Latency and *in planta* growth
  - Latent infections establishing at any growth stage between flowering and veraison can potentially persist through subsequent growth stages and lead to BBR symptoms at harvest.
  - Hyphal growth of *B. cinerea* in mature grape berries was localised between the cuticle and the epidermis.
- Symptom expression
  - Visual estimation of percentage BBR severity can be subject to significant inter-assessor variability.
  - Quantitative PCR is a highly accurate BBR quantification method, but may only be suitable as a research tool as it is too labour intensive.
  - NIR and mid-IR spectroscopy have potential as BBR quantification methods, but require further calibration in order to improve accuracy for visual BBR severities of $< 20\%$ and to ensure the method is repeatable.
  - The image analysis software, RotBot, was an accurate and repeatable method for assessing percentage BBR severity and required no specialised equipment.

In order to obtain a clearer understanding of the infection process and latency, particularly in relation to what happens in the vineyard, fungal growth needs to be observed from infection to symptom development. Flowers and bunches could be inoculated with GFP-expressing *B. cinerea* while still attached to living vines and samples collected at regular time points for the duration of the season. By sampling infected material at all

growth stages, the hyphal growth rate could be observed in real time using time-lapse confocal microscopy and related to the maturity of the grape berry. An experiment of this nature would require the use of containment facilities to allow compliance with regulations about the use of GMOs and this would probably limit the physical size of the trial. Mullins cuttings may be able to be used in place of full grown vines to reduce space required; however, the survival rate of the cuttings would need to be greater than was found in the glasshouse trial (Appendix D) and for the method to be considered reliable. Cross-contamination during inoculations would also need to be eliminated, either by shielding other treatments not being inoculated or inoculating cuttings in an area isolated from other cuttings.

A potential study investigating the link between latent infections and BBR severity at harvest could also provide a better understanding of infection timing and latent infection. Strain fitness and survival of latent infections could be investigated in relation to growth stage and the duration of the latent period could be determined. The premise would be similar to that used in the infection timing trials (Chapter 3). However, bunches could be inoculated at more frequent intervals, possibly as often as weekly, and the frequency of strains occurring as latent infections could be monitored regularly throughout the season. The *nit* mutant method may not be suitable for such a study as a large number of unique strains would be needed for inoculations, making the screening process too cumbersome. Identifying genetic markers that allowed the differentiation of unique strains could make the use of large numbers of unique strains in inoculations realistic. A molecular approach could also allow the screening of whole bunches to identify all *B. cinerea* strains present. Substantial genetic diversity has been identified in studies concerned with *B. cinerea* population genetics (Asadollahi *et al.*, 2013, Fekete *et al.*, 2012). Some of the unique isolates identified in these studies may be useful as traceable strains. The naturally occurring population would need to be assessed prior to conducting research in a particular region to ensure the strains used were not already present.

Both the development and comparison of BBR quantification methods is of potential use for all future research on BBR. Of the methods tested, RotBot appears to have the highest potential benefit for both research and commercial applications. Once the protocol for the method has been properly developed to allow for standardised, repeatable assessments, the software could be adapted for the appropriate platform and released to the wine industry. The method also demonstrates the usefulness of simple image analysis that does not rely on specialised imaging sensors or other dedicated equipment. It is possible that RotBot could be adapted for the assessment of other rots, such as the

orange-coloured Ripe Rot caused by *Colletotrichum* spp. and the blue/green-coloured rot caused by *Penicillium* spp. (Steel et al., 2013). The hue-based approach employed by RotBot may also be useful in other aspects of vineyard management, such as determining the stage of veraison in red grapes. It could also have applications in other crops and for other diseases. Any feature of a crop that can be assessed by the human eye could potentially be assessed by image analysis if distinguishing pixel characteristics can be identified.

Quantitative PCR is potentially the most useful quantification method for all BBR research applications and not just in relation to harvest severity. Future infection trials could incorporate qPCR throughout the season and relate the pathogen coefficient (PC) to percentage incidence from the ONFIT in order to determine whether a predictive relationship exists between the two variables. If a new method was used to quantify latent infections with the intention of predicting BBR severity at harvest, the relationship between latent infections and BBR severity would need to be better understood. It was demonstrated here that latent infection can occur at any stage in the season, which may explain why a predictive relationship between latent infection at a single stage early in the season and BBR severity has not previously been found (Beresford & Hill, 2008, Nair *et al.*, 1995).

Once a reliable quantification method has been developed, and the link between latent infection and harvest BBR severity fully understood, larger trials could be conducted that relate the degree of BBR in a vineyard to the quality of the wine produced. Different wines could be produced using various degrees of BBR severity, with and without intervention from the wine maker, and compared using sensory science. This could lead to the establishment of BBR thresholds that can be related directly to their effect on the wine.

The findings from this study have enhanced our understanding of the BBR disease cycle in terms of infection timing, *in planta* growth and BBR quantification. However, the future research described above will be necessary to expand on these findings. The ultimate goal would be to achieve a degree of understanding of *B. cinerea* and BBR whereby, at any point in the season, a grape grower could quantify the amount of BBR in any chosen vineyard block, predict BBR severity at harvest and interpret what that would mean for the resulting wine. Disease management could then be selected and matched to the desired result. Such precise disease management would require an in-depth understanding of the pathogen and disease development, not only once symptoms are observed, but throughout the season, from infection to detection and right through to objective and rapid quantification of BBR at harvest.

A

Culturing and storage techniques for *Botrytis cinerea*

A.1. Introduction

The thesis research required a large amount of culturing and storage of *Botrytis cinerea* Pers. strains, which is commonplace for many researchers working with this fungus. However, in order to ensure these techniques were optimised for research presented in Chapter 3, they were tested experimentally prior to the research being carried out.

It has been found that *B. cinerea* conidia retain some germination potential after being stored dry for 2.5 years at temperatures below 0°C (Gindro & Pezet, 2001). However, the evidence for conidial survival as an aqueous spore suspension is largely anecdotal. Additionally, suspending spores in various percentages of glycerol is often a method of storage despite it not being clear whether the glycerol is necessary.

Increased spore density has been found to decrease germination percentage due to the excretion of self-inhibitors by spores (Wang, 1985; Ueno *et al.*, 1997; Chitarra *et al.*, 2004).

Culturing of *B. cinerea* poses some risk of contamination, despite being a relatively fast-growing fungus *in vitro*. While sterile technique reduces the risk of contamination from fungi present in the laboratory air spora, there is still a contamination risk when isolating from field material, particularly when incubating that material in non-sterile conditions. The predominant fungal contaminant is *Rhizopus* spp., which is present in both vineyards and laboratories with an apparently faster growth rate than *B. cinerea*. There are selective media assist in isolating *B. cinerea* (Edwards & Seddon, 2001); however, these are complicated to prepare and labour intensive when conducting experiments requiring large quantities of media Petri plates.

The first objective of this study was to investigate the effect of water or various concentrations of glycerol as storage media on germination percentage after various periods of storage. The second objective of this study was to investigate the effect of spore density on germination percentage using data from the trials presented in Chapter 3. The third objective of this study was to investigate the addition of dicloran to growth media for the control of contamination by *Rhizopus* spp. in *B. cinerea* cultures.

A.2. Materials and Methods

A.2.1. Isolates and spore suspensions

A wild-type *B. cinerea* strain was isolated from a *Vitis vinifera* L. Sauvignon blanc vine in a research vineyard in Auckland, New Zealand. A wild-type *Rhizopus* spp. strain was isolated from a contaminated *B. cinerea* culture. Both strains were grown on MEA for 10 d. For preparation of spore suspensions, cultures of *B. cinerea* or *Rhizopus* spp. were grown on malt extract agar (MEA) for 7 d under fluorescent lights on a 12 h light/dark cycle. Aliquots of distilled water (dH_2O; 2 ml) were added to each plate and the spores were agitated with a sterile polypropylene spreader. The resulting spore suspension was transferred to a sterile 1.5 ml microfuge tube. Spore concentration was measured using a haemocytometer and the spore suspensions were diluted in dH_2O to 1 x 10^5 spores/ml.

A.2.2. Spore suspension storage

The survival at -20°C of *B. cinerea* spores stored in dH_2O and various percentages of glycerol in dH_2O was investigated over a period of 180 d.

A spore suspension of the wild-type *B. cinerea* strain was prepared as described above and divided into three sterile 15 ml tubes and diluted in either dH_2O or sterile glycerol (25% v/v) to achieve a concentration of approximately 1 x 10^5 spores per ml in dH_2O, 5% glycerol and 10% glycerol. Aliquots of 200 μl of each of the three spore suspensions were transferred into 30 individual 0.6 ml centrifuge tubes and stored at -20°C. Five tubes from each storage medium were removed after 0, 1, 7, 30, 90 and 180 d storage at -20°C. The contents of each tube were spread on 1.5% (w/v) water agar in individual Petri plates that were then incubated under fluorescent lights for 24–36 h. Each plate was viewed at 100x magnification with the aid of a stereo light microscope. The number of germinating and non-germinating spores within one field of view was recorded and the germination percentage was calculated.

This experiment was repeated with three replicates (tubes of spore suspensions) of the same wild-type strain. The effect of storage on the survival of the nitrate non-utilising (*nit*) mutant strains N2-1 and N3-1 used in the infection trials (Chapter 3) was also tested. For these strians, germination percentage was calculated and averaged for three fields of view per Petri plate.

Germination percentage data from both experiments were combined for analysis of variance (ANOVA) at each time point with strain as a blocking effect. Mean germination percentage for each strain after storage for 180 d from the second experiment was used in ANOVA with storage medium as a blocking effect.

A.2.3. Effect of spore density on germination percentage

Spore density and germination percentage data was collected as described in Chapter 3.

A.2.4. Controlling contamination of cultures

Spore suspensions were prepared from cultures of two *nit* strains of *B. cinerea*, N2-1 and N3-1, and one wild-type strain of *Rhizopus* spp. An aliquot of spore suspension (10 μl) was pipetted onto an MEA plate amended with 100 ppm streptomycin and 0, 2, 3 or 4 ppm dicloran (Botran 75 WP; DuPont, Wilmington, DE, USA). There were three replicate Petri plates per fungal strain and growth medium.

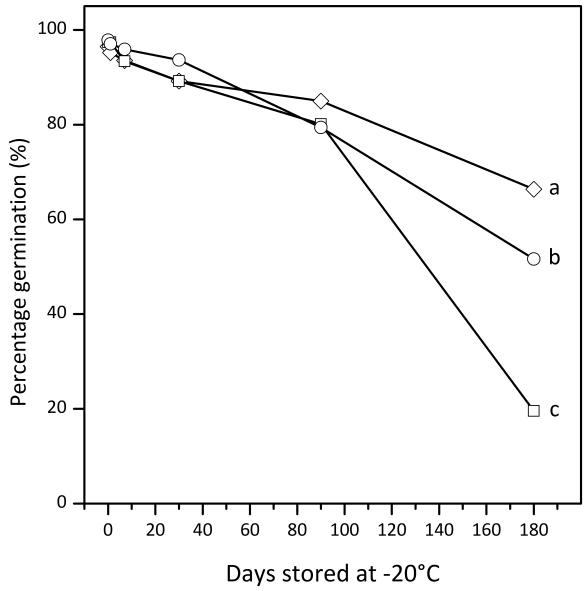

Figure A.1 *Botrytis cinerea* spore germination percentage after storage at -20°C in water (diamonds), 5% glycerol (squares) and 10% glycerol (circles). Data were combined from two experiments: one using only a single wild-type strain and the other using three strains (wild-type, N2-1 mutant and N3-1 mutant). Means at 0, 7, 30 or 90 d are not significantly different. Means at 180 d with the same letter are not significantly different ($P < 0.05$).

The diameter of each of the fungal colonies was measured with a ruler (1 mm increments) 1, 2 and 3 d after transfer of the spore suspension. A linear regression of colony diameter versus growth time was performed and the slope was used as the growth rate of each colony. Relative growth rate was then calculated for each strain as a percentage of the mean growth rate on MEA with 0 ppm dicloran.

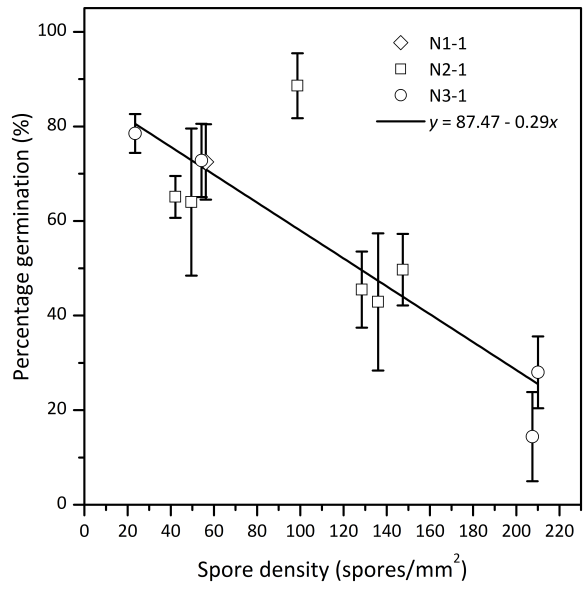

Figure A.2 Linear regression of spore density against mean germination percentage for *Botrytis cinerea* used in all inoculations in all trials conducted between 2010 and 2013 ($R^2 = 0.71$; $P < 0.001$). Regression line has been fitted to all data points. Error bars represent standard error (d.f. = 2).

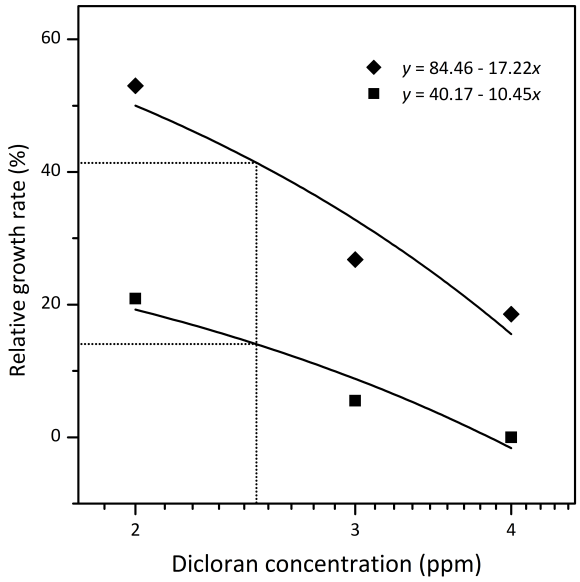

Figure A.3 Mean relative growth rate for strains of *Botrytis cinerea* (diamonds; $R^2 = 0.97$; $P < 0.001$) and wild-type *Rhizopus* spp. (squares; $R^2 = 0.99$; $P < 0.001$) colonies grown on malt extract agar containing various concentrations of dicloran. Growth rate is relative to growth on 0 ppm dicloran. Dotted lines indicate the relative growth rates of both fungi at the concentration of dicloran used to grow *B. cinerea* isolates (2.5 ppm). *X*-axis shown on a log scale.

A.3. Results

A.3.1. Effect of spore storage or density on germination percentage

Germination percentages of *B. cinerea* conidia stored at -20°C declined over time for all storage media tested (Figure A.1). There were no significant differences ($P < 0.05$) among storage media for germination percentage after 0, 7, 30 or 90 d. Mean germination rate was significantly different ($P < 0.05$) among all storage media after 180 d, with water retaining the highest germination percentage. There were no significant differences ($P < 0.05$) among strains for germination percentage after 180 d.

A significant negative relationship ($P < 0.05$) was found between the mean germination percentage of spores used in inoculations and spore density (Figure A.2).

A.3.2. Controlling *Rhizopus* spp. contamination of *Botrytis cinerea* cultures

Rhizopus spp. growth was inhibited more than *B. cinerea* when grown on MEA amended with dicloran (Figure A.3). This allowed *B. cinerea* to outgrow *Rhizopus* spp. contamination and produce enough spores to make the necessary spore suspension. A dicloran concentration of 2.5 ppm was used to culture *B. cinerea* isolates prior to screening.

Based on linear regression of dicloran concentration against relative growth rate, this concentration reduced the growth rate of *Rhizopus* spp. to 14.1% of wild-type growth and the growth rate of *B. cinerea* to 41.6% of wild-type growth.

A.4. Conclusions

This study showed that water was not only sufficient for storage of *B. cinerea* spores for up to 180 days, it was the most effective storage medium in terms of retaining germination potential. While germination rates dropped steadily from the time the spore suspensions were stored in the freezer, significant differences in the means of the various storage liquids were not detected until they had been stored for 180 days. After 180 days, spores stored in water had the highest percentage germination, while spores stored in 5% glycerol had the lowest. It is unclear why storage in 5% glycerol should result in a lower germination rate than storage in water, or why storage in 10% should have a lesser effect. Glycerol has been shown to reduce germination rate in *Fusarium graminearum* when introduced to growth media, however this effect was similar or greater when the concentration was increased (Ramirez *et al.*, 2004). Further work needs to investigate the effect of storage liquid on *in vitro* growth and pathogenicity.

A dicloran concentration of 2.5 ppm added to MEA was sufficient to control *Rhizopus* spp. contamination in B. cinerea cultures. Spore suspensions of adequate concentrations were still obtained from *B. cinerea* cultures grown on MEA amended with dicloran, despite the reduction in *B. cinerea* hyphal growth.

B

Polycyclic infection trial

B.1. Introduction

Botrytis bunch rot (BBR), a disease of grapes caused by the fungus *Botrytis cinerea* Pers., is considered a polycyclic disease (Elmer & Michailides 2004), meaning that an infected bunch becomes a source of inoculum for infection to occur in neighbouring bunches. While the mechanism for polycyclic infection exists, the disease epidemic is restricted by season length, so it is possible that there is insufficient time for polycyclic secondary infection to occur in any significant capacity. In addition to this, airborne inoculum levels throughout the vineyard may be so high (Rodríguez-Rajo *et al.*, 2010) that the inoculum from neighbouring bunches has no significant impact on the spread of the disease.

The objective of this study was to investigate the influence of bunches exhibiting BBR symptoms as an inoculum source for the infection of neighbouring bunches.

B.2. Materials and Methods

B.2.1. Trial layout

A trial was carried out at a *Vitis vinifera* L. Sauvignon blanc research vineyard in Auckland, New Zealand (-37.2038, 174.8636). Four vine rows were used as blocks, each separated by one row of non-treated vines. Blocks consisted of three plots of 4–6 vines with two non-treated vines between each plot. Each plot within each block was assigned to one of three treatments: 1) non-treated control, 2) symptomatic bunches removed from vines following the first BBR incidence assessment, and 3) symptomatic bunches removed from vine following each BBR incidence assessment.

B.2.2. Disease assessments and bunch removal

The total number of bunches in each plot was recorded on 20 March 2012.

The presence of *B. cinerea* sporulation was recorded for 25 bunches per plot for Treatments 1 and 2 on 20 March 2012, 29 March 2012 and 05 April 2012 and for all remaining bunches in each plot on 26 April 2012.

All bunches with *B. cinerea* sporulation were removed following bunch assessments on 20 March 2012 for Treatments 2 and 3 and on 29 March 2012 and 05 April 2012 for Treatment 3. The total number of bunches removed from the vine was recorded for each plot. Bunches were removed from the vineyard to eliminate them as an inoculum source.

B.2.3. Botrytis bunch rot incidence

BBR incidence was calculated differently for each treatment as the number of bunches removed was not the same for all treatments.

BBR incidence for Treatment 1 (non-treated control) was calculated for each plot as the percentage of assessed bunches in that plot with *B. cinerea* sporulation. On 20 March 2012 for Treatments 2 and on all assessment dates for Treatment 3, BBR incidence was calculated as the total number of bunches removed in each plot as a percentage of the initial total number of bunches for that plot. On 29 March 2012 and 05 April 2012, BBR

Treatment	BBR Incidence
Non-treated control	62.8
Bunches removed once	59.4
Bunches removed three times	70.9

Table B.1 Mean botrytis bunch rot (BBR) incidence for all treatments on 20 April 2012. Means were not significantly different (P > 0.05).

incidence for Treatment 2 was calculated as for Treatment 1 and corrected to account for bunches removed on 20 March 2013 using the following formula:

$$f(x) = I_i + \frac{I_x(100 - I_i)}{100} \tag{B.1}$$

where I_i = BBR incidence on 20 March 2012 and I_x = BBR incidence calculated on 29 March 2012 or 05 April 2012.

Analysis of variance (ANOVA) was performed using mean BBR incidence for each plot for each treatment on each assessment date.

B.3. Results

Mean BBR incidence over the season did not increase uniformly for all treatments (Figure B.1). There were no significant differences ($P > 0.05$) in mean BBR incidence between any of the treatments at the final assessment on 26 April 2012 (Table B.1).

On 20 March 2012, mean BBR incidence for Treatment 1 was significantly higher ($P < 0.05$) than the other two treatments. On 29 March 2012, mean BBR incidence for Treatments 1 and 2 was significantly higher ($P < 0.05$) than Treatment 3.

B.4. Conclusions

Reducing inoculum by removing bunches from vines did not affect BBR incidence at the final assessment. This suggests that polycyclic infection may not play a significant role in BBR disease spread and that latent infections occurring earlier in the season

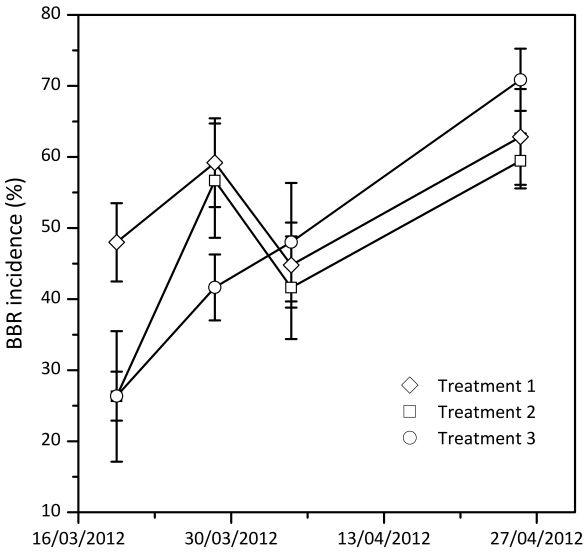

Figure B.1 Mean botrytis bunch rot (BBR) incidence for non-treated vines (Treatment 1), vines with symptomatic bunches removed on 20 March 2012 (Treatment 2) and vines with symptomatic bunches removed on 20 March 2012, 29 March 2012 and 05 April 2012 (Treatment 3). Incidence was calculated differently for each treatment at each date. Error bars represent standard error.

have a much larger influence on increases in disease intensity. It is also possible that in the act of assessing bunches and removing those with sporulation, spores may have been spread from bunch to bunch via contact. This could have increased BBR incidence and masked any decrease resulting from the removal of bunches. Without accurate, uniform measurements of incidence throughout the season it is not possible to make any conclusions about whether or not this is the case. Differences in the calculation of BBR incidence amongst treatments and on different assessment dates make direct comparisons of treatment means unreliable.

Mean BBR incidence differed between Treatments 1 and the other two treatments on the first assessment. At this assessment, only a sub-sample of bunches in each plot were assessed for Treatment 1, whereas all bunches were assessed for the other two treatments. The differences seen at the first assessment suggest that the differences in sample size and incidence calculations were not comparable as there should have been no differences in these treatments at this stage. The sample size of 25 bunches may not be sufficient for estimating BBR incidence within a plot of the size used in this trial.

This trial provides some evidence that polycyclic infection does not play a significant role in the increase in BBR incidence within a vineyard. This would mean that infection earlier in the season, resulting in latent infection, is the predominant source of infection. This conclusion is based on the assumption that spread is more likely to occur between neighbouring bunches. Secondary spread from bunches in neighbouring rows or from

elsewhere in the vineyard may be considerable enough that the removal of nearby inoculum sources is not enough to reduce the overall inoculum present.

Future experiments may need to account for inoculum sources outside individual plots. This might be achieved by spraying buffer vines and rows with botryticides to reduce the overall inoculum load or by removing all bunches from outside the experimental plots. A more accurate assessment of BBR incidence throughout the season, e.g. assessing all bunches within each plot, would also allow for better comparison between treatments.

C

Quantification of botrytis bunch rot using laccase assays

C.1. Introduction

Much of the concern surrounding botrytis bunch rot (BBR) is due to the laccase enzyme produced by *Botrytis cinerea* Pers. present in the must. A laccase assay allows the activity of the laccase enzyme to be measured directly and the amount of *B. cinerea* to be inferred from this estimate (Grassin and Dubourdieu 1989). One of the shortcomings of this method is its inability to work in the presence sulphur dioxide (SO_2), which is used to sterilise the must. While some correlations have been found between laccase activity and infection by *B. cinerea* (Roudet *et al.* 1992, Dewey *et al.* 2008), evidence for the laccase assay to accurately quantify *B. cinerea* is weak.

The objective of this experiment was to assess three different laccase assays using samples of berries from bunches assessed visually for BBR severity (Chapter 6).

C.2. Materials and methods

C.2.1. Samples

Berries were collected from the *Vitis vinifera* L. Reisling samples used in the comparison of quantification methods (Chapter 6). Approximately 100 berries were collected from each sample of bunches with average severities of 0, 5, 10, 25, 50 and 78%. Berries were placed in a sterile plastic container and crushed in the containers using another, smaller container. The juice was poured into 50 ml centrifuge tubes and stored at -20°C for 14 d. Tubes were then placed on dry ice and transported by air to the laboratory where the laccase assays were carried out.

C.2.2. Laccase assays

Juice samples were centrifuged and the supernatant used in laccase assays carried out by Adrian Coulter and Gayle Baldock of the Australian Wine Research Institute (AWRI, Glen Osmond, South Australia). Two commercially available laccase assays and one laboratory-based chemical assay were used: (1) Dolmar Laccase Detection Kit (Dolmar, Haro, Spain), (2) Botrytest (Laffort, Bordeaux, France) and (3) 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS). The ABTS assay was repeated using a 1:1 dilution for the 25, 50 and 70% juice samples in sterile water. Assay methodology remains the property of the AWRI.

Linear regressions were performed on mean BBR severity (%) for the severity group against laccase activity (units/ml) as measured by each assay.

C.3. Results

Significant relationships ($P < 0.05$) were found between BBR severity and laccase activity for all assays (Figure C.1). All three assays measured laccase activity as units/ml, but used a different scale for units. The Dolmar Laccase Detection Kit gave the highest R^2 of the two commercially available assays (Figure C.1a). Both the Dolmar Laccase Detection Kit and the Botrytest gave the same results for the two juice samples from the 50% and 78% groups (Figure C.1a,b). The ABTS assay could only distinguish the

two juice samples from the 50% and 78% groups with dilution of the original samples (Figure C.1c).

C.4. Conclusions

All three laccase assays were able to quantify BBR in the juice samples when the severity was $\leq 50\%$. As lower BBR severities ($< 25\%$) are more common in vineyards, it is not entirely relevant that the higher severity groups were not differentiated. However, the ABTS assay gave the most accurate predictions of BBR when the original samples were diluted. The diluted samples were not used with the two commercially available assays, but it is possible that dilution may have also improved their predictions of BBR.

There was no replication in this study as juice samples were pooled for each severity group. Therefore, it is difficult to draw conclusions as to the repeatability and accuracy of these assays. Further testing with replicated samples and more severity groups at lower severities ($< 25\%$) would be required to confirm the accuracy of these assays.

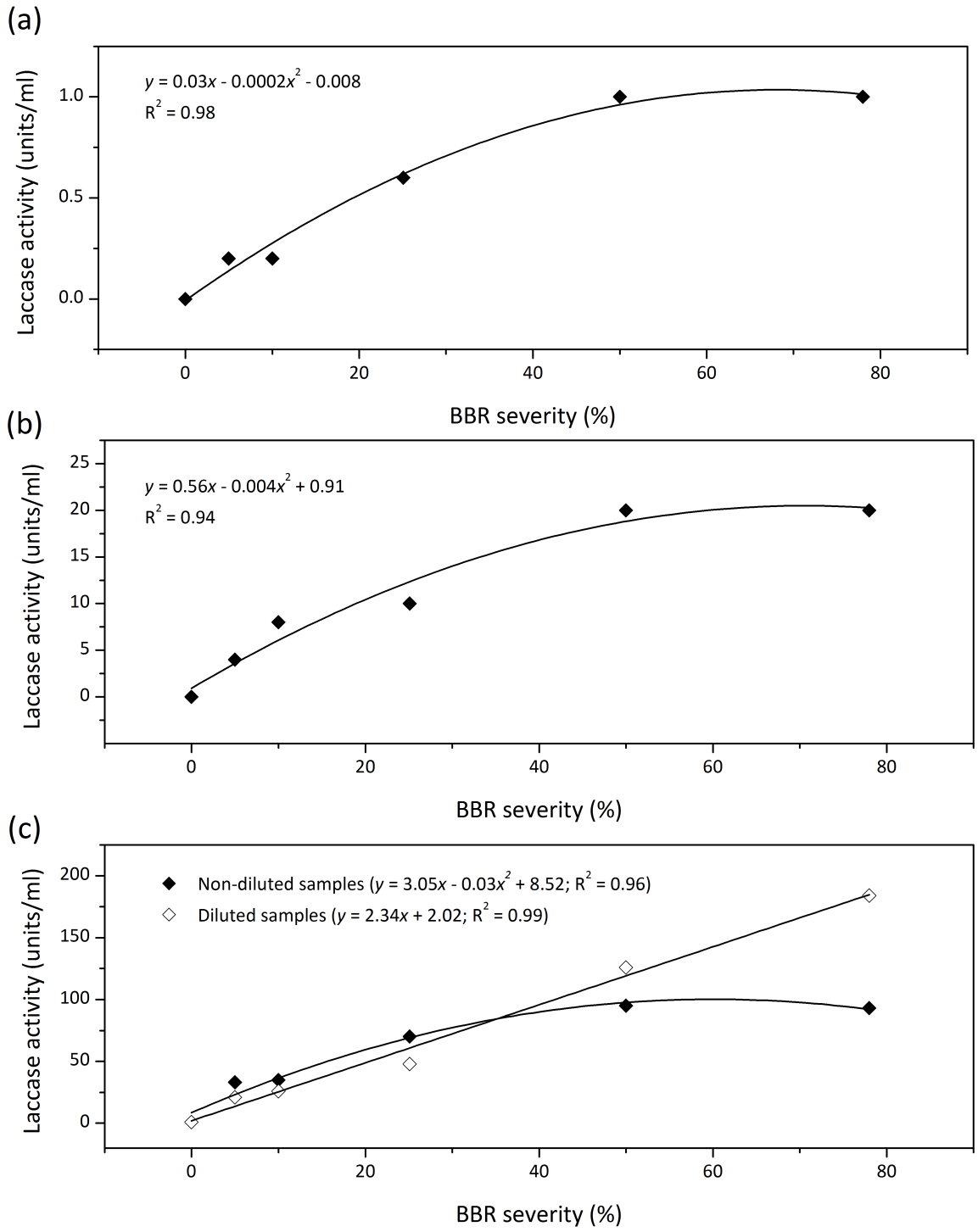

Figure C.1. Linear regressions of mean botrytis bunch rot (BBR) severity against laccase activity measured by the Dolmar Laccase Detection Kit (a), Botrytest (b) and 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid (ABTS) assay with and without dilution (c).

D

Mullins cuttings survival rates

D.1. Introduction

Flowering *Vitis vinifera* L. grapevine cuttings, referred to as Mullins cuttings, are single node cuttings that produce a grape bunch similar to that found in the vineyard (Mullins and Rajasekaran 1981). The success rate of these cuttings is variable and can depend on the variety used (Mullins & Rajasekaran 1981, Lebon *et al.* 2005).

The objective of this experiment was to determine the success rate of Mullins cuttings from *V. vinifera* Pinot Noir and Sauvignon Blanc vines used in the glasshouse trial.

D.2. Materials and methods

The initial number of vine cuttings prepared was 540 of each of Pinot noir and Sauvignon blanc. Refer to Chapter 3 for detail on how Mullins cuttings were prepared and used in the glasshouse trial.

Table D.1. Percentage bud burst and root growth for Pinot noir ($n = 395$) and Sauvignon blanc ($n = 414$) Mullins cutting prepared for the glasshouse trial that did not produce inflorescences.

	Bud burst evident		No bud burst	
Variety	Roots present	Roots absent	Roots present	Roots absent
Pinot noir	48.4	6.8	21.3	23.5
Sauvignon blanc	39.6	1.2	47.8	11.4

Cuttings were considered successful if an inflorescence was produced. These cuttings did not necessarily survive beyond the inflorescence stage.

Failed cuttings were observed for the development of roots (> 1 mm) and whether or not buds had burst.

D.3. Results

The success rate of Mullins cuttings was 26.9% for Pinot noir and 23.3% for Sauvignon blanc. The presence or absence of root growth was not recorded for successful cuttings.

Of the failed cuttings, 48.4% of the Pinot noir and 39.6% of the Sauvignon blanc cuttings produced roots and went through bud burst but did not produce an inflorescence (Table D.1). A lack of root growth was only observed in 21.3% of the failed cuttings.

D.4. Conclusions

The failure of Mullins cuttings to produce an inflorescence did not appear to be related to the presence or absence of root growth. The majority of cuttings that went through bud burst developed roots; however, a high percentage of cuttings with roots did not go through bud burst. The majority of Sauvignon blanc cuttings with roots present did not go through bud burst.

These results do not explain why such a high percentage of cuttings failed to develop inflorescences. There may have been other factors contributing to this phenomenon. As no other factors were examined, it is not possible to determine the underlying cause. The success rate of Mullins cuttings needs to improve if they are to be used in future experiments, particularly if treatments are applied before it is known whether a cutting is viable.

E

C# code for RotBot software

E.1. Program.cs

```
using System;
using System.Collections.Generic;
using System.Linq;
using System.Windows.Forms;

namespace RotBot
{
    static class Program
    {
        [STAThread]
        static void Main()
        {
            Application.EnableVisualStyles();
            Application.SetCompatibleTextRenderingDefault(false);
            Application.Run(new Form1());
        }
    }
}
```

E.2. Form1.designer.cs

```
namespace RotBot
{
    partial class Form1
    {

        private System.ComponentModel.IContainer components = null;

        protected override void Dispose(bool disposing)
        {
            if (disposing && (components != null))
            {
                components.Dispose();
            }
            base.Dispose(disposing);
        }

        #region Windows Form Designer generated code

        private void InitializeComponent()
        {
            System.ComponentModel.ComponentResourceManager resources =
               new System.ComponentModel.ComponentResourceManager(
               typeof(Form1));
            this.pictureBox1 = new System.Windows.Forms.PictureBox();
            this.btnLoadImage = new System.Windows.Forms.Button();
            this.openFileDialog1 = new System.Windows.Forms.
               OpenFileDialog();
            this.btnCalculate = new System.Windows.Forms.Button();
            this.progressBar1 = new System.Windows.Forms.ProgressBar();
            this.btnOriginalImg = new System.Windows.Forms.Button();
            this.btnExit = new System.Windows.Forms.Button();
            this.btnImAGEd = new System.Windows.Forms.Button();
            this.lblBBR = new System.Windows.Forms.Label();
            this.numMaxOther = new System.Windows.Forms.NumericUpDown()
               ;
            this.numMinOther = new System.Windows.Forms.NumericUpDown()
               ;
            this.numMaxPlant = new System.Windows.Forms.NumericUpDown()
               ;
            this.lblMaxHue = new System.Windows.Forms.Label();
            this.numMinPlant = new System.Windows.Forms.NumericUpDown()
               ;
            this.lblMinHue = new System.Windows.Forms.Label();
            this.lblPlant = new System.Windows.Forms.Label();
            this.lblOther = new System.Windows.Forms.Label();
            this.btnReset = new System.Windows.Forms.Button();
            this.btnSave = new System.Windows.Forms.Button();
            this.panel1 = new System.Windows.Forms.Panel();
            this.lblFile = new System.Windows.Forms.Label();
            this.panel2 = new System.Windows.Forms.Panel();
            this.lblSmooth = new System.Windows.Forms.Label();
            this.numSmooth = new System.Windows.Forms.NumericUpDown();
            this.btnClipboard = new System.Windows.Forms.Button();
            this.pnlHue = new System.Windows.Forms.Panel();
```

```
this.progressBar2 = new System.Windows.Forms.ProgressBar();
this.checkBox1 = new System.Windows.Forms.CheckBox();
this.btnInvisible = new System.Windows.Forms.Button();
this.lblAdjusted = new System.Windows.Forms.Label();
((System.ComponentModel.ISupportInitialize)(this.
    pictureBox1)).BeginInit();
((System.ComponentModel.ISupportInitialize)(this.
    numMaxOther)).BeginInit();
((System.ComponentModel.ISupportInitialize)(this.
    numMinOther)).BeginInit();
((System.ComponentModel.ISupportInitialize)(this.
    numMaxPlant)).BeginInit();
((System.ComponentModel.ISupportInitialize)(this.
    numMinPlant)).BeginInit();
this.panel1.SuspendLayout();
this.panel2.SuspendLayout();
((System.ComponentModel.ISupportInitialize)(this.numSmooth)
    ).BeginInit();
this.pnlHue.SuspendLayout();
this.SuspendLayout();
//
// pictureBox1
//
this.pictureBox1.AllowDrop = true;
this.pictureBox1.BackColor = System.Drawing.SystemColors.
    Window;
this.pictureBox1.BorderStyle = System.Windows.Forms.
    BorderStyle.Fixed3D;
this.pictureBox1.Image = ((System.Drawing.Image)(resources.
    GetObject("pictureBox1.Image")));
this.pictureBox1.Location = new System.Drawing.Point(195,
    12);
this.pictureBox1.Name = "pictureBox1";
this.pictureBox1.Size = new System.Drawing.Size(394, 556);
this.pictureBox1.SizeMode = System.Windows.Forms.
    PictureBoxSizeMode.AutoSize;
this.pictureBox1.TabIndex = 0;
this.pictureBox1.TabStop = false;
this.pictureBox1.DragDrop += new System.Windows.Forms.
    DragEventHandler(this.pictureBox1_DragDrop);
this.pictureBox1.DragEnter += new System.Windows.Forms.
    DragEventHandler(this.pictureBox1_DragEnter);
//
// btnLoadImage
//
this.btnLoadImage.Location = new System.Drawing.Point(9, 7)
    ;
this.btnLoadImage.Name = "btnLoadImage";
this.btnLoadImage.Size = new System.Drawing.Size(161, 37);
this.btnLoadImage.TabIndex = 1;
this.btnLoadImage.Text = "Load Image";
this.btnLoadImage.UseVisualStyleBackColor = true;
this.btnLoadImage.Click += new System.EventHandler(this.
    btnLoadImage_Click_1);
```

```
            //
            // openFileDialog1
            //
            this.openFileDialog1.FileName = "*.jpg";
            this.openFileDialog1.Filter = "Image Files (*.jpeg; *.jpg;
               *.bmp; *.gif; *.png)|*.jpeg; *.jpg; *.png; *.gif; *.b"
               +
    "mp|All Files (*.*)|*.*";
            //
            // btnCalculate
            //
            this.btnCalculate.Enabled = false;
            this.btnCalculate.Location = new System.Drawing.Point(9,
               50);
            this.btnCalculate.Name = "btnCalculate";
            this.btnCalculate.Size = new System.Drawing.Size(161, 37);
            this.btnCalculate.TabIndex = 2;
            this.btnCalculate.Text = "Calculate Severity";
            this.btnCalculate.UseVisualStyleBackColor = true;
            this.btnCalculate.Click += new System.EventHandler(this.
               btnSimplify_Click);
            //
            // progressBar1
            //
            this.progressBar1.Location = new System.Drawing.Point(9, 6)
               ;
            this.progressBar1.Name = "progressBar1";
            this.progressBar1.Size = new System.Drawing.Size(161, 62);
            this.progressBar1.Style = System.Windows.Forms.
               ProgressBarStyle.Continuous;
            this.progressBar1.TabIndex = 3;
            this.progressBar1.Visible = false;
            //
            // btnOriginalImg
            //
            this.btnOriginalImg.Enabled = false;
            this.btnOriginalImg.Location = new System.Drawing.Point(9,
               136);
            this.btnOriginalImg.Name = "btnOriginalImg";
            this.btnOriginalImg.Size = new System.Drawing.Size(161, 37)
               ;
            this.btnOriginalImg.TabIndex = 4;
            this.btnOriginalImg.Text = "Original";
            this.btnOriginalImg.UseVisualStyleBackColor = true;
            this.btnOriginalImg.Click += new System.EventHandler(this.
               btnOriginalImg_Click_1);
            //
            // btnExit
            //
            this.btnExit.Location = new System.Drawing.Point(9, 442);
            this.btnExit.Name = "btnExit";
            this.btnExit.Size = new System.Drawing.Size(161, 37);
            this.btnExit.TabIndex = 5;
            this.btnExit.Text = "Exit";
```

```
this.btnExit.UseVisualStyleBackColor = true;
this.btnExit.Click += new System.EventHandler(this.
    btnExit_Click);
//
// btnImAGEd
//
this.btnImAGEd.Enabled = false;
this.btnImAGEd.Location = new System.Drawing.Point(9, 93);
this.btnImAGEd.Name = "btnImAGEd";
this.btnImAGEd.Size = new System.Drawing.Size(161, 37);
this.btnImAGEd.TabIndex = 6;
this.btnImAGEd.Text = "Simplified";
this.btnImAGEd.UseVisualStyleBackColor = true;
this.btnImAGEd.Click += new System.EventHandler(this.
    btnSimpleImg_Click);
//
// lblBBR
//
this.lblBBR.AutoSize = true;
this.lblBBR.BackColor = System.Drawing.Color.White;
this.lblBBR.Font = new System.Drawing.Font("Calibri", 14F,
    System.Drawing.FontStyle.Bold);
this.lblBBR.ForeColor = System.Drawing.Color.Black;
this.lblBBR.Location = new System.Drawing.Point(5, 28);
this.lblBBR.Name = "lblBBR";
this.lblBBR.Size = new System.Drawing.Size(43, 23);
this.lblBBR.TabIndex = 9;
this.lblBBR.Text = "BBR";
this.lblBBR.Visible = false;
//
// numMaxOther
//
this.numMaxOther.Location = new System.Drawing.Point(116,
    48);
this.numMaxOther.Maximum = new decimal(new int[] {
360,
0,
0,
0});
this.numMaxOther.Name = "numMaxOther";
this.numMaxOther.Size = new System.Drawing.Size(45, 20);
this.numMaxOther.TabIndex = 165;
this.numMaxOther.Value = new decimal(new int[] {
45,
0,
0,
0});
//
// numMinOther
//
this.numMinOther.Location = new System.Drawing.Point(116,
    22);
this.numMinOther.Maximum = new decimal(new int[] {
360,
```

```
0,
0,
0});
this.numMinOther.Name = "numMinOther";
this.numMinOther.Size = new System.Drawing.Size(45, 20);
this.numMinOther.TabIndex = 166;
this.numMinOther.Value = new decimal(new int[] {
1,
0,
0,
0});
//
// numMaxPlant
//
this.numMaxPlant.Location = new System.Drawing.Point(65,
    48);
this.numMaxPlant.Maximum = new decimal(new int[] {
360,
0,
0,
0});
this.numMaxPlant.Name = "numMaxPlant";
this.numMaxPlant.Size = new System.Drawing.Size(45, 20);
this.numMaxPlant.TabIndex = 164;
this.numMaxPlant.Value = new decimal(new int[] {
75,
0,
0,
0});
//
// lblMaxHue
//
this.lblMaxHue.AutoSize = true;
this.lblMaxHue.Location = new System.Drawing.Point(8, 50);
this.lblMaxHue.Name = "lblMaxHue";
this.lblMaxHue.Size = new System.Drawing.Size(54, 13);
this.lblMaxHue.TabIndex = 162;
this.lblMaxHue.Text = "Maximum:";
//
// numMinPlant
//
this.numMinPlant.Location = new System.Drawing.Point(65,
    22);
this.numMinPlant.Maximum = new decimal(new int[] {
360,
0,
0,
0});
this.numMinPlant.Name = "numMinPlant";
this.numMinPlant.Size = new System.Drawing.Size(45, 20);
this.numMinPlant.TabIndex = 163;
this.numMinPlant.Value = new decimal(new int[] {
46,
0,
```

```
0,
0});
//
// lblMinHue
//
this.lblMinHue.AutoSize = true;
this.lblMinHue.Location = new System.Drawing.Point(8, 24);
this.lblMinHue.Name = "lblMinHue";
this.lblMinHue.Size = new System.Drawing.Size(51, 13);
this.lblMinHue.TabIndex = 161;
this.lblMinHue.Text = "Minimum:";
//
// lblPlant
//
this.lblPlant.AutoSize = true;
this.lblPlant.Location = new System.Drawing.Point(62, 6);
this.lblPlant.Name = "lblPlant";
this.lblPlant.Size = new System.Drawing.Size(43, 13);
this.lblPlant.TabIndex = 167;
this.lblPlant.Text = "Healthy";
this.lblPlant.Click += new System.EventHandler(this.
    lblPlant_Click);
//
// lblOther
//
this.lblOther.AutoSize = true;
this.lblOther.Location = new System.Drawing.Point(113, 6);
this.lblOther.Name = "lblOther";
this.lblOther.Size = new System.Drawing.Size(46, 13);
this.lblOther.TabIndex = 168;
this.lblOther.Text = "Infected";
this.lblOther.Click += new System.EventHandler(this.
    lblOther_Click);
//
// btnReset
//
this.btnReset.Enabled = false;
this.btnReset.Location = new System.Drawing.Point(9, 179);
this.btnReset.Name = "btnReset";
this.btnReset.Size = new System.Drawing.Size(161, 37);
this.btnReset.TabIndex = 169;
this.btnReset.Text = "Reset";
this.btnReset.UseVisualStyleBackColor = true;
this.btnReset.Click += new System.EventHandler(this.
    btnReset_Click);
//
// btnSave
//
this.btnSave.Enabled = false;
this.btnSave.Location = new System.Drawing.Point(9, 356);
this.btnSave.Name = "btnSave";
this.btnSave.Size = new System.Drawing.Size(161, 37);
this.btnSave.TabIndex = 170;
this.btnSave.Text = "Save Current Image";
```

```
this.btnSave.UseVisualStyleBackColor = true;
this.btnSave.Click += new System.EventHandler(this.
    btnSave_Click);
//
// panel1
//
this.panel1.BackColor = System.Drawing.SystemColors.Window;
this.panel1.Controls.Add(this.lblFile);
this.panel1.Controls.Add(this.lblAdjusted);
this.panel1.Controls.Add(this.lblBBR);
this.panel1.Location = new System.Drawing.Point(9, 485);
this.panel1.Name = "panel1";
this.panel1.Size = new System.Drawing.Size(161, 79);
this.panel1.TabIndex = 172;
this.panel1.Visible = false;
//
// lblFile
//
this.lblFile.AutoSize = true;
this.lblFile.BackColor = System.Drawing.Color.White;
this.lblFile.Font = new System.Drawing.Font("Calibri", 14F,
     System.Drawing.FontStyle.Bold);
this.lblFile.ForeColor = System.Drawing.Color.Black;
this.lblFile.Location = new System.Drawing.Point(5, 5);
this.lblFile.Name = "lblFile";
this.lblFile.Size = new System.Drawing.Size(83, 23);
this.lblFile.TabIndex = 11;
this.lblFile.Text = "Filename";
this.lblFile.Visible = false;
//
// panel2
//
this.panel2.Controls.Add(this.lblSmooth);
this.panel2.Controls.Add(this.numSmooth);
this.panel2.Controls.Add(this.btnClipboard);
this.panel2.Controls.Add(this.pnlHue);
this.panel2.Controls.Add(this.checkBox1);
this.panel2.Controls.Add(this.btnExit);
this.panel2.Controls.Add(this.panel1);
this.panel2.Controls.Add(this.btnSave);
this.panel2.Controls.Add(this.btnReset);
this.panel2.Controls.Add(this.btnImAGEd);
this.panel2.Controls.Add(this.btnOriginalImg);
this.panel2.Controls.Add(this.btnCalculate);
this.panel2.Controls.Add(this.btnLoadImage);
this.panel2.Controls.Add(this.btnInvisible);
this.panel2.Location = new System.Drawing.Point(10, 12);
this.panel2.Name = "panel2";
this.panel2.Size = new System.Drawing.Size(179, 572);
this.panel2.TabIndex = 173;
//
// lblSmooth
//
this.lblSmooth.AutoSize = true;
```

```
this.lblSmooth.Location = new System.Drawing.Point(30, 332)
    ;
this.lblSmooth.Name = "lblSmooth";
this.lblSmooth.Size = new System.Drawing.Size(60, 13);
this.lblSmooth.TabIndex = 176;
this.lblSmooth.Text = "Smoothing:";
//
// numSmooth
//
this.numSmooth.Location = new System.Drawing.Point(96, 330)
    ;
this.numSmooth.Maximum = new decimal(new int[] {
30,
0,
0,
0});
this.numSmooth.Minimum = new decimal(new int[] {
1,
0,
0,
0});
this.numSmooth.Name = "numSmooth";
this.numSmooth.Size = new System.Drawing.Size(45, 20);
this.numSmooth.TabIndex = 169;
this.numSmooth.Value = new decimal(new int[] {
5,
0,
0,
0});
//
// btnClipboard
//
this.btnClipboard.Enabled = false;
this.btnClipboard.Location = new System.Drawing.Point(9,
    399);
this.btnClipboard.Name = "btnClipboard";
this.btnClipboard.Size = new System.Drawing.Size(161, 37);
this.btnClipboard.TabIndex = 175;
this.btnClipboard.Text = "Copy to Clipboard";
this.btnClipboard.UseVisualStyleBackColor = true;
this.btnClipboard.Click += new System.EventHandler(this.
    btnClipboard_Click);
//
// pnlHue
//
this.pnlHue.Controls.Add(this.progressBar2);
this.pnlHue.Controls.Add(this.progressBar1);
this.pnlHue.Controls.Add(this.numMinPlant);
this.pnlHue.Controls.Add(this.lblOther);
this.pnlHue.Controls.Add(this.lblMinHue);
this.pnlHue.Controls.Add(this.lblPlant);
this.pnlHue.Controls.Add(this.lblMaxHue);
this.pnlHue.Controls.Add(this.numMaxOther);
this.pnlHue.Controls.Add(this.numMaxPlant);
```

```
this.pnlHue.Controls.Add(this.numMinOther);
this.pnlHue.Enabled = false;
this.pnlHue.Location = new System.Drawing.Point(0, 222);
this.pnlHue.Name = "pnlHue";
this.pnlHue.Size = new System.Drawing.Size(179, 77);
this.pnlHue.TabIndex = 174;
//
// progressBar2
//
this.progressBar2.Location = new System.Drawing.Point(9, 6)
    ;
this.progressBar2.Name = "progressBar2";
this.progressBar2.Size = new System.Drawing.Size(161, 62);
this.progressBar2.TabIndex = 174;
this.progressBar2.Visible = false;
//
// checkBox1
//
this.checkBox1.AutoSize = true;
this.checkBox1.Checked = true;
this.checkBox1.CheckState = System.Windows.Forms.CheckState
    .Checked;
this.checkBox1.Location = new System.Drawing.Point(56, 307)
    ;
this.checkBox1.Name = "checkBox1";
this.checkBox1.Size = new System.Drawing.Size(71, 17);
this.checkBox1.TabIndex = 173;
this.checkBox1.Text = "Auto Hue";
this.checkBox1.UseVisualStyleBackColor = true;
this.checkBox1.CheckedChanged += new System.EventHandler(
    this.checkBox1_CheckedChanged);
//
// btnInvisible
//
this.btnInvisible.Location = new System.Drawing.Point(9,
    50);
this.btnInvisible.Name = "btnInvisible";
this.btnInvisible.Size = new System.Drawing.Size(161, 37);
this.btnInvisible.TabIndex = 174;
this.btnInvisible.Text = "You can\'t see me";
this.btnInvisible.UseVisualStyleBackColor = true;
this.btnInvisible.Visible = false;
//
// lblAdjusted
//
this.lblAdjusted.AutoSize = true;
this.lblAdjusted.BackColor = System.Drawing.Color.White;
this.lblAdjusted.Font = new System.Drawing.Font("Calibri",
    14F, System.Drawing.FontStyle.Bold);
this.lblAdjusted.ForeColor = System.Drawing.Color.Black;
this.lblAdjusted.Location = new System.Drawing.Point(5, 51)
    ;
this.lblAdjusted.Name = "lblAdjusted";
this.lblAdjusted.Size = new System.Drawing.Size(82, 23);
```

```
            this.lblAdjusted.TabIndex = 10;
            this.lblAdjusted.Text = "Adjusted";
            this.lblAdjusted.Visible = false;
            //
            // Form1
            //
            this.AllowDrop = true;
            this.AutoScaleDimensions = new System.Drawing.SizeF(6F, 13F
                );
            this.AutoScaleMode = System.Windows.Forms.AutoScaleMode.
                Font;
            this.AutoSize = true;
            this.AutoSizeMode = System.Windows.Forms.AutoSizeMode.
                GrowAndShrink;
            this.ClientSize = new System.Drawing.Size(600, 594);
            this.Controls.Add(this.panel2);
            this.Controls.Add(this.pictureBox1);
            this.FormBorderStyle = System.Windows.Forms.FormBorderStyle
                .Fixed3D;
            this.Icon = ((System.Drawing.Icon)(resources.GetObject("
                $this.Icon")));
            this.MaximizeBox = false;
            this.MinimizeBox = false;
            this.Name = "Form1";
            this.SizeGripStyle = System.Windows.Forms.SizeGripStyle.
                Hide;
            this.StartPosition = System.Windows.Forms.FormStartPosition
                .CenterScreen;
            this.Text = "RotBot";
            this.Load += new System.EventHandler(this.Form1_Load);
            ((System.ComponentModel.ISupportInitialize)(this.
                pictureBox1)).EndInit();
            ((System.ComponentModel.ISupportInitialize)(this.
                numMaxOther)).EndInit();
            ((System.ComponentModel.ISupportInitialize)(this.
                numMinOther)).EndInit();
            ((System.ComponentModel.ISupportInitialize)(this.
                numMaxPlant)).EndInit();
            ((System.ComponentModel.ISupportInitialize)(this.
                numMinPlant)).EndInit();
            this.panel1.ResumeLayout(false);
            this.panel1.PerformLayout();
            this.panel2.ResumeLayout(false);
            this.panel2.PerformLayout();
            ((System.ComponentModel.ISupportInitialize)(this.numSmooth)
                ).EndInit();
            this.pnlHue.ResumeLayout(false);
            this.pnlHue.PerformLayout();
            this.ResumeLayout(false);
            this.PerformLayout();

        }

        #endregion
```

```
        private System.Windows.Forms.PictureBox pictureBox1;
        private System.Windows.Forms.Button btnLoadImage;
        private System.Windows.Forms.OpenFileDialog openFileDialog1;
        private System.Windows.Forms.Button btnCalculate;
        private System.Windows.Forms.ProgressBar progressBar1;
        private System.Windows.Forms.Button btnOriginalImg;
        private System.Windows.Forms.Button btnExit;
        private System.Windows.Forms.Button btnImAGEd;
        private System.Windows.Forms.Label lblBBR;
        private System.Windows.Forms.NumericUpDown numMaxOther;
        private System.Windows.Forms.NumericUpDown numMinOther;
        private System.Windows.Forms.NumericUpDown numMaxPlant;
        private System.Windows.Forms.Label lblMaxHue;
        private System.Windows.Forms.NumericUpDown numMinPlant;
        private System.Windows.Forms.Label lblMinHue;
        private System.Windows.Forms.Label lblPlant;
        private System.Windows.Forms.Label lblOther;
        private System.Windows.Forms.Button btnReset;
        private System.Windows.Forms.Button btnSave;
        private System.Windows.Forms.Panel panel1;
        private System.Windows.Forms.Panel panel2;
        private System.Windows.Forms.CheckBox checkBox1;
        private System.Windows.Forms.Panel pnlHue;
        private System.Windows.Forms.Button btnClipboard;
        private System.Windows.Forms.Label lblFile;
        private System.Windows.Forms.NumericUpDown numSmooth;
        private System.Windows.Forms.Label lblSmooth;
        private System.Windows.Forms.ProgressBar progressBar2;
        private System.Windows.Forms.Button btnInvisible;
        private System.Windows.Forms.Label lblAdjusted;
    }
}
```

E.3. Form 1.cs

```
using System;
using System.Collections.Generic;
using System.ComponentModel;
using System.Data;
using System.Drawing;
using System.Linq;
using System.Text;
using System.Windows.Forms;
using System.IO;

namespace RotBot
{
    public partial class Form1 : Form
    {
        public Form1()
        {
```

```
        InitializeComponent();
    }

    Bitmap image;
    Bitmap original;
    Bitmap simpleImage;
    Color diseaseColour;
    Color vineColour;
    Color otherColour;
    int filecount;
    string BBR;
    string adjBBR;
    string file;
    bool autoHue = true;

    private void btnLoadImage_Click_1(object sender, EventArgs e)
    {
        if (openFileDialog1.ShowDialog() == DialogResult.OK)
        {
            file = openFileDialog1.FileName;

            LoadPicture lp = new LoadPicture();
            lp.setImage(file, pictureBox1, btnCalculate, btnImAGEd,
                btnOriginalImg, lblFile, panel1);
            image = new Bitmap(lp.getSimplified());
            original = new Bitmap(lp.getOriginal());

            btnSave.Enabled = true;
            btnClipboard.Enabled = true;
        }

    }

    private void pictureBox1_DragEnter(object sender, DragEventArgs
        e)
    {
        if (e.Data.GetDataPresent(DataFormats.FileDrop, false))
        {
            e.Effect = DragDropEffects.All;
        }
    }

    private void pictureBox1_DragDrop(object sender, DragEventArgs
       e)
    {
        string[] files = (string[])e.Data.GetData(DataFormats.
           FileDrop, false);

        filecount = files.Count();

        if (filecount == 1)
        {
            file = files[0].ToLower();
```

```
        if (file.EndsWith(".jpg") || file.EndsWith(".jpeg") ||
            file.EndsWith(".bmp") || file.EndsWith(".png") ||
            file.EndsWith(".gif"))
        {

            LoadPicture lp = new LoadPicture();
            lp.setImage(file, pictureBox1, btnCalculate,
                btnImAGEd, btnOriginalImg, lblFile, panel1);
            image = new Bitmap(lp.getSimplified());
            original = new Bitmap(lp.getOriginal());
            Application.DoEvents();

            btnSave.Enabled = true;
            btnClipboard.Enabled = true;
        }
    }

    if (filecount > 1)
    {
        BatchProcess bp = new BatchProcess();

        btnLoadImage.Enabled = false;

        bp.setSaveFolder();
        bp.setArray(filecount);

        progressBar2.Visible = true;
        progressBar2.Minimum = 0;
        progressBar2.Maximum = filecount;

        for (int i = 0; i < files.Count(); i++)
        {
            string file = files[i].ToLower();
            string filename = Path.GetFileNameWithoutExtension(
                files[i]);

            if (file.EndsWith(".jpg") || file.EndsWith(".jpeg")
                || file.EndsWith(".bmp") || file.EndsWith(".
                png") || file.EndsWith(".gif"))
            {

                if (bp.GetCancel() != true)
                {

                    LoadPicture lp = new LoadPicture();
                    lp.setImage(file, pictureBox1, btnInvisible
                        , btnImAGEd, btnOriginalImg, lblFile,
                        panel1);
                    image = new Bitmap(lp.getSimplified());
                    original = new Bitmap(lp.getOriginal());

                    Application.DoEvents();

                    try
```

```
                    {
                        bp.getFormParts(progressBar1,
                            pictureBox1, image,Convert.ToInt32(
                            numSmooth.Value));

                        bp.calcBBR(filename, i);

                        progressBar2.Value = i + 1;
                    }

                    catch
                    {
                        MessageBox.Show("An error has occurred
                            with this image.", "Error",
                            MessageBoxButtons.OK,
                            MessageBoxIcon.Error);
                    }
                }
            }
        }

        progressBar2.Visible = false;

        try
        {
            bp.saveFile();
            string meanBBR = bp.getMeanBBR();
            if (MessageBox.Show("RotBot Analysis complete. Mean
                 BBR is "+meanBBR+"%. \r\nWould you like open
                the spreadsheet?", "RotBot Analysis Complete",
                MessageBoxButtons.YesNo, MessageBoxIcon.
                Question) == DialogResult.Yes)
                 bp.openFile();
        }

        catch
        {
            MessageBox.Show("An error has occurred with RotBot
                calculations.", "Error", MessageBoxButtons.OK,
                MessageBoxIcon.Error);
        }

    }
}

private void btnSimplify_Click(object sender, EventArgs e)
{
    btnCalculate.Enabled = false;

    HueChange hc = new HueChange();
    hc.getFormParts(progressBar1, pictureBox1, image);

    diseaseColour = Color.Indigo;
    vineColour = Color.LimeGreen;
```

```
            otherColour = Color.Black;

            hc.setSmoothing(Convert.ToInt32(numSmooth.Value));

            hc.hueCounter();
            hc.setBackground();
            hc.hueShift();
            hc.percentHue();

            hc.setMinHue();
            hc.setMaxHue();
            hc.setPeaks();
            hc.setTrough();

            hc.setRanges();

            if (autoHue == true)
            {
                numMinOther.Value = hc.getDiseaseMin();
                numMaxOther.Value = hc.getDiseaseMax();
                numMinPlant.Value = hc.getHealthyMin();
                numMaxPlant.Value = hc.getHealthyMax();
            }

            if (autoHue == false)
                hc.manualRanges(int.Parse(numMinOther.Text), int.Parse(
                    numMaxOther.Text), int.Parse(numMinPlant.Text), int
                    .Parse(numMaxPlant.Text));

            hc.hueChanger(diseaseColour, vineColour, otherColour);

            hc.setPixelPercent();
            simpleImage = hc.getSimpleImage();
            BBR = hc.getBBR();
            adjBBR = hc.getAdjBBR();

            lblBBR.Text = "BBR: " + BBR + "%";
            lblBBR.Visible = true;

            panel1.Visible = true;

            btnSave.Enabled = true;
            pictureBox1.Image = simpleImage;

            btnImAGEd.Enabled = true;
            btnOriginalImg.Enabled = true;

            btnReset.Enabled = true;

            btnImAGEd.Font = new Font(btnImAGEd.Font, FontStyle.Bold);
            btnOriginalImg.Font = new Font(btnOriginalImg.Font,
                FontStyle.Regular);

        }
```

```
private void btnExit_Click(object sender, EventArgs e)
{
    Application.Exit();
}

private void btnOriginalImg_Click_1(object sender, EventArgs e)
{
    pictureBox1.Image = original;
    btnOriginalImg.Font = new Font(btnOriginalImg.Font,
       FontStyle.Bold);
    btnImAGEd.Font = new Font(btnImAGEd.Font, FontStyle.Regular
       );
}

private void btnSimpleImg_Click(object sender, EventArgs e)
{
    pictureBox1.Image = simpleImage;
    btnImAGEd.Font = new Font(btnImAGEd.Font, FontStyle.Bold);
    btnOriginalImg.Font = new Font(btnOriginalImg.Font,
       FontStyle.Regular);
}

private void lblPlant_Click(object sender, EventArgs e)
{
    InputBox ib = new InputBox();
    ib.SetText(lblPlant.Text);
    ib.ShowDialog();
    lblPlant.Text = ib.GetText();
}

private void lblOther_Click(object sender, EventArgs e)
{
    InputBox ib = new InputBox();
    ib.SetText(lblOther.Text);
    ib.ShowDialog();
    lblOther.Text = ib.GetText();
}

private void btnReset_Click(object sender, EventArgs e)
{
    LoadPicture lp = new LoadPicture();
    lp.setImage(file, pictureBox1, btnCalculate, btnImAGEd,
       btnOriginalImg, lblFile, panel1);
    image = new Bitmap(lp.getSimplified());
    original = new Bitmap(lp.getOriginal());

    btnOriginalImg.Font = new Font(btnOriginalImg.Font,
       FontStyle.Regular);
    btnImAGEd.Font = new Font(btnImAGEd.Font, FontStyle.Regular
       );

    btnReset.Enabled = false;
}
```

```
private void btnSave_Click(object sender, EventArgs e)
{
    SaveFileDialog save = new SaveFileDialog();
    save.Title = "Save current image";
    save.Filter = "Jpeg (*.jpg)|*.jpg|Portable Network Graphic
       (*.png)|*.png|Bitmap (*.bmp)|*.bmp";

    if (save.ShowDialog() == DialogResult.OK)
    {
        pictureBox1.Image.Save(save.FileName.ToString());
    }
}

private void Form1_Load(object sender, EventArgs e)
{
    lblPlant.Text = Properties.Settings.Default.lblPlant;
    lblOther.Text = Properties.Settings.Default.lblOther;

    numMinPlant.Value = Properties.Settings.Default.minPlant;
    numMaxPlant.Value = Properties.Settings.Default.maxPlant;
    numMinOther.Value = Properties.Settings.Default.minOther;
    numMaxOther.Value = Properties.Settings.Default.maxOther;
}

private void btnDefaults_Click(object sender, EventArgs e)
{
    lblPlant.Text = Properties.DefaultSettings.Default.lblPlant
       ;
    lblOther.Text = Properties.DefaultSettings.Default.lblOther
       ;

    numMinPlant.Value = Properties.DefaultSettings.Default.
       minPlant;
    numMaxPlant.Value = Properties.DefaultSettings.Default.
       maxPlant;
    numMinOther.Value = Properties.DefaultSettings.Default.
       minOther;
    numMaxOther.Value = Properties.DefaultSettings.Default.
       maxOther;
}

private void checkBox1_CheckedChanged(object sender, EventArgs
   e)
{
    if (checkBox1.Checked == true)
    {
        autoHue = true;
        pnlHue.Enabled = false;

    }

    if (checkBox1.Checked == false)
    {
```

```
                autoHue = false;
                pnlHue.Enabled = true;
            }
        }

        private void btnClipboard_Click(object sender, EventArgs e)
        {
            Clipboard.SetImage(pictureBox1.Image);
        }

    }
}
```

E.4. LoadPicture.cs

```
using System;
using System.Drawing;
using System.IO;
using System.Windows.Forms;

namespace RotBot
{
    class LoadPicture
    {
        private Bitmap loadImage;
        private Bitmap image;
        private Bitmap original;
        private PictureBox pictureBox1;
        private Button btnCalculate;
        private Button btnImAGEd;
        private Button btnOriginal;
        private Label lblFile;
        private Panel panel1;

        private int imageWidth;
        private int imageHeight;

        private int maxHeight = 572;

        public void setImage(string imagefile, PictureBox pb, Button
            imageify, Button imaged, Button orig, Label file, Panel pnl
            )
        {
            loadImage = new Bitmap(imagefile);
            pictureBox1 = pb;
            btnCalculate = imageify;
            btnImAGEd = imaged;
            btnOriginal = orig;
            panel1 = pnl;
            lblFile = file;

            if (loadImage.Height >= maxHeight)
```

```
            {
                imageHeight = maxHeight;
                double dblWidth = loadImage.Width * imageHeight /
                    loadImage.Height;
                imageWidth = Convert.ToInt32(dblWidth);
            }
            else
            {
                imageWidth = loadImage.Width;
                imageHeight = loadImage.Height;
            }

            image = new Bitmap(loadImage, imageWidth, imageHeight);
            original = new Bitmap(loadImage, imageWidth, imageHeight);

            pictureBox1.Image = image;
            btnCalculate.Enabled = true;
            btnImAGEd.Enabled = false;
            btnOriginal.Enabled = false;
            panel1.Visible = false;

            lblFile.Text = Path.GetFileNameWithoutExtension(imagefile);
            lblFile.Visible = true;

        }

        public Bitmap getSimplified()
        {
            return image;
        }

        public Bitmap getOriginal()
        {
            return original;
        }
    }
}
```

E.5. HueChange.cs

```
using System;
using System.Linq;
using System.Windows.Forms;
using System.Drawing;
using System.Text;
using System.IO;

namespace RotBot
{
    class HueChange
    {
        private ProgressBar progressBar1;
```

```
private Bitmap image;
private PictureBox pictureBox1;
private double pxlHealthy;
private double pxlDisease;
private double pxlOther;
private string percentVine;
private string percentDisease;
private double BBR;
private int[] hueArray = new int[361];
private int[] modHueArray = new int[361];
private double[] pcntArray = new double[361];
private int trough;
private int maxPeakHue;
private int penultPeakHue;
private int peakDif;
private int disPeak;
private int healthPeak;
private int count = 0;
private int imageSize;
private int maxHue;
private int minHue;
private int diseaseMin;
private int diseaseMax;
private int healthyMin;
private int healthyMax;
private int backgrndMin = 180;
private int backgrndMax = 270;
private int shiftValue = 90;
private int excludeThrshld;
private int defaultTrough = 35;
private int meanSmoothing = 5;

public void getFormParts(ProgressBar pbar, PictureBox pbox,
   Bitmap bmp)
{
    progressBar1 = pbar;
    pictureBox1 = pbox;
    image = bmp;

    imageSize = image.Width * image.Height;
    count = 0;

    progressBar1.Minimum = 0;
    progressBar1.Maximum = imageSize;
}

public void hueCounter()
{
    for (int y = 0; y < image.Height; y++)
    {
        for (int x = 0; x < image.Width; x++)
        {
            double hue = image.GetPixel(x, y).GetHue();
```

```
                hueArray[Convert.ToInt32(hue)]++;

                count++;

                Application.DoEvents();
            }
        }
    }

    public void setBackground()
    {
        backgrndMin = 180;
        backgrndMax = 270;
        shiftValue = 90;
        excludeThrshld = 270;

        defaultTrough = defaultTrough + shiftValue;
    }

    public void hueShift()
    {
        for (int i = 1; i < hueArray.Count() - 1; i++)
        {

            if (i >= backgrndMin && i <= backgrndMax)
                continue;

            if (i >= backgrndMax) //shifting values of hues to make
                all potential disease and infected hues continuous
                modHueArray[i - backgrndMax] = hueArray[i];

            if (i < backgrndMax)
                modHueArray[i + shiftValue] = hueArray[i];
        }

        modHueArray[shiftValue] = (modHueArray[shiftValue - 1] +
            modHueArray[shiftValue + 1]) / 2; //removes 0 value for
             what was hue = 0 as it interferred with trough
            calculations
    }

    public void setPeaks()
    {
        for (int i = minHue; i < excludeThrshld; i++)
        {
            if (pcntArray[i] == pcntArray.Max())
                maxPeakHue = i;
        }

        peakDif = Convert.ToInt32(maxPeakHue * 0.2);

        for (int i = minHue + peakDif; i < excludeThrshld - peakDif
            ; i++)
        {
```

```
            if (pcntArray[i] < pcntArray.Max() && pcntArray[i] >
                pcntArray[penultPeakHue] && Math.Abs(maxPeakHue - i
                ) > peakDif) //peakDif check required to remove
                values within peak.
            {
                if (pcntArray[i] > pcntArray[i - peakDif] &&
                    pcntArray[i] > pcntArray[i + peakDif])
                    penultPeakHue = i;
                else
                    continue;
            }
        }

        if (maxPeakHue > penultPeakHue && maxPeakHue >
            defaultTrough)
        {
            healthPeak = maxPeakHue;
            disPeak = penultPeakHue;
        }

        if (maxPeakHue > penultPeakHue && maxPeakHue <
            defaultTrough)
        {
            disPeak = maxPeakHue;
            healthPeak = defaultTrough + 1;
        }

        if (maxPeakHue < penultPeakHue && maxPeakHue <
            defaultTrough)
        {
            disPeak = maxPeakHue;
            healthPeak = penultPeakHue;
        }

        if (maxPeakHue > defaultTrough && penultPeakHue >
            defaultTrough)
        {
            healthPeak = maxPeakHue;
            disPeak = minHue + 1;
        }

    }
    public void percentHue()
    {
        double hueSum = modHueArray.Sum();

        for (int i = 0; i < modHueArray.Count() - meanSmoothing; i
            ++) //creates moving average to smooth data
        {

            float[] smoothArray = new float[meanSmoothing * 2];

            int smooth = 0;
```

```
            for (int j = -meanSmoothing; j < meanSmoothing; j++)
            {
                if (i + j < 0)
                    continue;

                smoothArray[smooth] = modHueArray[i + j];

                smooth++;
            }

            pcntArray[i] = (smoothArray.Average() / hueSum * 100);
        }
    }


    public int setMinHue()
    {
        minHue = 1;

        return minHue;
    }

    public int setMaxHue()
    {
        maxHue = excludeThrshld;

        return maxHue;
    }


    public void setTrough()
    {
        defaultTrough = healthPeak - peakDif;

        int troughChk = 5;

        for (int i = minHue + troughChk; i < maxHue - troughChk; i
            ++)
        {
            if (pcntArray[i] < pcntArray[i - troughChk] &&
                pcntArray[i] < pcntArray[i + troughChk] && i <
                healthPeak)
                trough = i;
        }

        if (trough > defaultTrough || trough < disPeak)
            trough = defaultTrough;
    }

    public void setRanges()
    {
        diseaseMin = minHue;
        diseaseMax = trough;
```

```
        healthyMin = trough + 1;
        healthyMax = maxHue;
    }

    public void manualRanges(int disMin, int disMax, int healthMin,
        int healthMax)
    {
        diseaseMin = disMin;
        diseaseMax = disMax;

        healthyMin = healthMin;
        healthyMax = healthMax;
    }

    public void hueChanger(Color disease, Color vine, Color other)
    {
        progressBar1.Minimum = 0;
        progressBar1.Maximum = imageSize;

        pxlHealthy = 0;
        pxlDisease = 0;
        pxlOther = 0;

        count = 0;

        int changeCounter = 0;

        for (int y = 0; y < image.Height; y++)
        {
            for (int x = 0; x < image.Width; x++)
            {
                count++;

                int hue = Convert.ToInt32(image.GetPixel(x, y).
                    GetHue());

                if (hue == 0)
                {
                    image.SetPixel(x, y, other);
                    pxlOther++;
                    continue;
                }

                if (hue >= backgrndMax)
                    hue = hue - backgrndMax;
                else if (hue < backgrndMax)
                    hue = hue + shiftValue;

                if (hue >= diseaseMin && hue <= diseaseMax)
                {
                    image.SetPixel(x, y, disease);
                    pxlDisease++;
                }
```

```
                if (hue >= healthyMin && hue <= healthyMax)
                {
                    image.SetPixel(x, y, vine);
                    pxlHealthy++;
                }

                if (hue < diseaseMin || hue > healthyMax)
                {
                    image.SetPixel(x, y, other);
                    pxlOther++;
                }

                changeCounter++;

                if (changeCounter > image.Width)
                {
                    pictureBox1.Image = image;
                    Application.DoEvents();
                    changeCounter = 0;
                }

            }
        }

        progressBar1.Visible = false;
        progressBar1.Value = 0;
    }

    public void setPixelPercent()
    {
        percentVine = (pxlHealthy / (pxlHealthy + pxlDisease +
            pxlOther) * 100).ToString("#0") + "%";
        percentDisease = (pxlDisease / (pxlHealthy + pxlDisease +
            pxlOther) * 100).ToString("#0") + "%";
        BBR = pxlDisease / (pxlHealthy + pxlDisease) * 100;
    }

    public Bitmap getSimpleImage()
    {
        return image;
    }

    public string getPercentVine()
    {
        return percentVine;
    }

    public string getPercentDisease()
    {
        return percentDisease;
    }

    public string getBBR()
    {
```

```
            return Math.Round(BBR, 0).ToString("#0");
        }

        public int getDiseaseMin()
        {
            if (diseaseMin > diseaseMax)
                diseaseMin = 1;

            return diseaseMin;
        }

        public int getDiseaseMax()
        {
            return diseaseMax;
        }

        public int getHealthyMin()
        {
            return healthyMin;
        }

        public int getHealthyMax()
        {
            if (healthyMax < healthyMin)
                healthyMax = healthyMin + 1;

            return healthyMax;
        }

        public void setSmoothing(int smooth)
        {
            meanSmoothing = smooth;
        }

    }
}
```

E.6. BatchProcess.cs

```
using System;
using System.Collections.Generic;
using System.Linq;
using System.Text;
using System.IO;
using System.Windows.Forms;
using System.Drawing;

namespace RotBot
{
    class BatchProcess
    {
        private ProgressBar progressBar1;
```

```
private Bitmap image;
private Bitmap simpleImage;
private PictureBox pictureBox1;
private string savefolder;
private bool cancel = false;
private int imageSize;
private string newDirectory;
private string savefile;
private double[] BBRArray;
private double[] adjBBRArray;
private string[] filenameArray;
private string allText;
private StringBuilder sb = new StringBuilder();
private int filecount;
private int smooth;
private Color diseaseColour;
private Color vineColour;
private Color otherColour;

public void setSaveFolder()
{
    FolderBrowserDialog fb = new FolderBrowserDialog();
    fb.Description = "Select Save Folder";
    if (fb.ShowDialog() != DialogResult.Cancel)
    {
        newDirectory = fb.SelectedPath;
        Directory.CreateDirectory(newDirectory);
        savefolder = newDirectory;
    }
    else
        cancel = true;

    string time = DateTime.Now.Hour.ToString() + DateTime.Now.
       Minute.ToString();

    savefile = savefolder + "/RotBot_" + time + ".csv";
}

public bool GetCancel()
{
    return cancel;
}

public void getFormParts(ProgressBar pbar, PictureBox pbox,
   Bitmap bmp, int s)
{
    progressBar1 = pbar;
    pictureBox1 = pbox;
    image = bmp;
    smooth = s;

    imageSize = image.Width * image.Height;
}
```

```
public void setArray(int fc)
{
    filecount = fc;

    filenameArray = new string[fc];

    BBRArray = new double[filecount];

    adjBBRArray = new double[filecount];
}

public void calcBBR(string f, int i)
{
    filenameArray[i] = f;

    HueChange hc = new HueChange();

    hc.getFormParts(progressBar1, pictureBox1, image);

    diseaseColour = Color.Indigo;
    vineColour = Color.LimeGreen;
    otherColour = Color.Black;

    hc.setSmoothing(smooth);
    hc.hueCounter();
    hc.setBackground();
    hc.hueShift();
    hc.percentHue();

    hc.setMinHue();
    hc.setMaxHue();
    hc.setPeaks();
    hc.setTrough();

    hc.setRanges();

    hc.hueChanger(diseaseColour, vineColour, otherColour);
    hc.setPixelPercent();
    simpleImage = hc.getSimpleImage();

    BBRArray[i] = double.Parse(hc.getBBR());
    adjBBRArray[i] = double.Parse(hc.getAdjBBR());
}

public string getMeanBBR()
{
    double meanBBR = BBRArray.Average();

    return Math.Round(meanBBR, 1).ToString("#.#");
}

public void saveFile()
{
    sb.AppendLine("Filename, BBR");
```

```
            for (int i = 0; i < filecount; i++)
            {
                sb.AppendLine(filenameArray[i] + "," + BBRArray[i]);
            }

            allText = sb.ToString();

            File.WriteAllText(savefile, allText);
        }

        public void openFile()
        {
            System.Diagnostics.Process.Start(savefile);
        }

    }
}
```

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